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STABILITY STUDIES OF LIPOSOMES AS A FUNCTION OF LIPID COMPOSITION, LIPOSOMAL TYPE, SURFACE CHARGE AND STORAGE CONDITIONS

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ABSTRACT

The effect of lipid composition, liposomal type, surface charge and storage conditions (time and temperature) on their stability was studied . The time course of lipid peroxidation for different liposomal formulations was chemically followed , during different storage conditions, using the thiobarbituric acid assay . Multilamellar and small unilamellar liposomes were prepared from egg lecithin and varying amounts of cholesterol with or without a charge inducing agent . Dicetyl phosphate or stearylamine was used to impart either a negative or a positive surface charge to liposomes. The results showed that, incorporation of cholesterol into the liposomal bilayer structure, generally, reduced the rate of lipid peroxidation and adjusting its content to 33 mole% could provide an appropriate molar ratio for maximum stability of liposomal system. Also, comparing between the two liposomal types of the same charge and storage conditions, small unilamellar liposomes showed significantly higher peroxidation values than multilamellar type . Regarding the liposomal surface charge, negatively charged liposomes showed the lowest peroxidation values and neutral liposomes showed the highest values, as compared to others of the same type and storage conditions. Thus, inclusion of charged lipid results in an structral stabilizing effect, which could be attributed to the steric barrier on the liposomal surface presented by this charge . In addition, concerning the effect of storage temperature on lipid peroxidation, liposomes showed higher stability when stored at low temperature than when stored at high temperature . Finally, for choosing the proper storage conditions to optimize liposomal stability freeze-dried and freeze-thawed liposomes were prepared and compared with untreated liposomes . Freeze-dried negatively charged multilamellar liposomes could provide an optimal storage condition for maintaining liposomal stability . In conclusion, it should be stressed that lipsomal lipid composition, type, surface charge and storage conditions can be modified in a variety of ways, and so can potentially be tailored to satisfy the needs of the formulation for solving the stability problems of liposomes .

INTRODUCTION

Artificial phospholipid vesicles or liposomes have been widly used both as a model membrane system for biological membrane and as controlled delivery system for therapeutic agents. Compared to other drug carriers, liposomes can be considered to be one specific type of drug carrier which has certain advantages as they composed of materials that are present as natural materials in the human body [1-3]. Therefore, they are non-toxic, biocompatible, biodegradable, and their size and surface can be modified or controlled to alter their biodistribution and pharmacokinetics[4].

However, it should be emphasized that liposomes face a number of chemical and physical destabilization process. These small vesicle structures were generally not thermodynamically stable and have a tendency to aggregate and fuse . For these reasons, the most important aspect in the use of liposomes as drug carrier is their stability .

Generally, the basic lipid composition of liposomal formulations consists of a mixture of phospholipids and cholesterol . Phospholipids usually form the backbone of the liposomal bilayer structure and their chemical stability is important. Also, incorporation of cholesterol into liposomal phsopholipid membranes provide a stabilizing effect on the bilayer configuration of lipids and thus reduce transbilayer movement . It was reported that, cholesterol intercalats or fills the empty spaces among the phospholipid molecules, where the 3-ß-hydroxyl on the steroid ring of cholesterol interacts with the carbonyl oxygens of the phospholipids [5,6] .

Many studies demonstrated that, liposomes were susceptible to peroxidation and hydrolysis within few months and were not likely to be stable at room temperature. Lipid peroxidation of liposomal phospholipids tends to generate potentially toxic oxidative degradation products with a highly different chemical nature.

In addition, lipid peroxidation causes an increase in the permeability of the lipid bilayer structure, and this would leads to destabilization of the membrane integrity of liposomes [2,4,5] .

Lipid peroxidation process involves the direct reaction of oxygen and lipid to form free radical intermediates and to produce semistable peroxides. Because of their unpaired electrons, free radicals react very energetically and initiate relatively nonspecific hydrogen abstraction and chemical addition reactions. The degree of peroxidation or the amount of lipid peroxidation products was determined as malondialdehyde formation[7-9].

Malondialdehyde, formed as the breakdown product produced during lipid peroxidation, serves as a convenient index for determining the extent of the peroxidation reaction and was measured by the thiobarbituric acid reaction[10]. It has been known that, the thiobarbituric acid assay is a useful tool in monitoring lipid peroxidation and is the most frequently used index of lipid peroxidation both in vitro and in vivo because it is extremly sensitive and simple [10-13] .

The object of this study was to investigate the effect of lipid composition, charge and vesicle type of liposomes on their stability during various storage conditions. The design and optimization of new methodology for providing stable liposomes or extending the shelf-life of their preparation were the aim we dealt with in this study .

Probably, it should be emphasized that, even small changes in lipid composition and methodological details can lead to substantial differences in the properties of the resultant liposomes . Therefore, different types of liposomal formulations were prepared and the stability during storage was carfully followed using the thiobarbituric acid test.

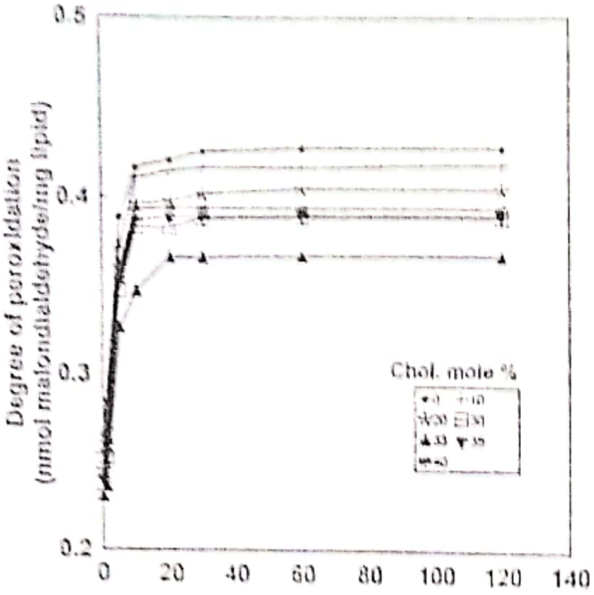

Fig.(1): Effect of cholesterol content of liposomal membranes on lipid peroxidation of multilamellar liposomal formulations consisting of phosphatidylcholine and cholesterol (up to 40 mole %) and incubated for different times(0,1,5,10,20,30,60 and 120 days) at 25 °C.

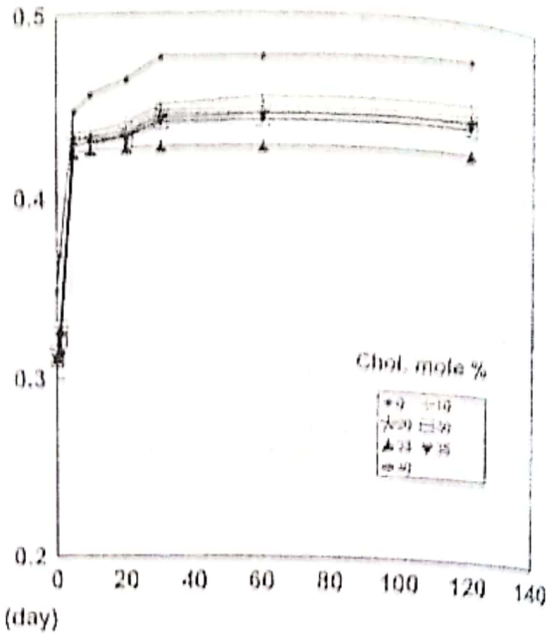

Fig.(2): Effect of cholesterol content of liposomal membranes on lipid peroxidation of small unilamellar liposomal formulations consisting of phosphatidylcholine and cholesterol (up to 40 mole %) and incubated for different times(0,1,5,10,20,30,60 and 120 days) at 25 °C.

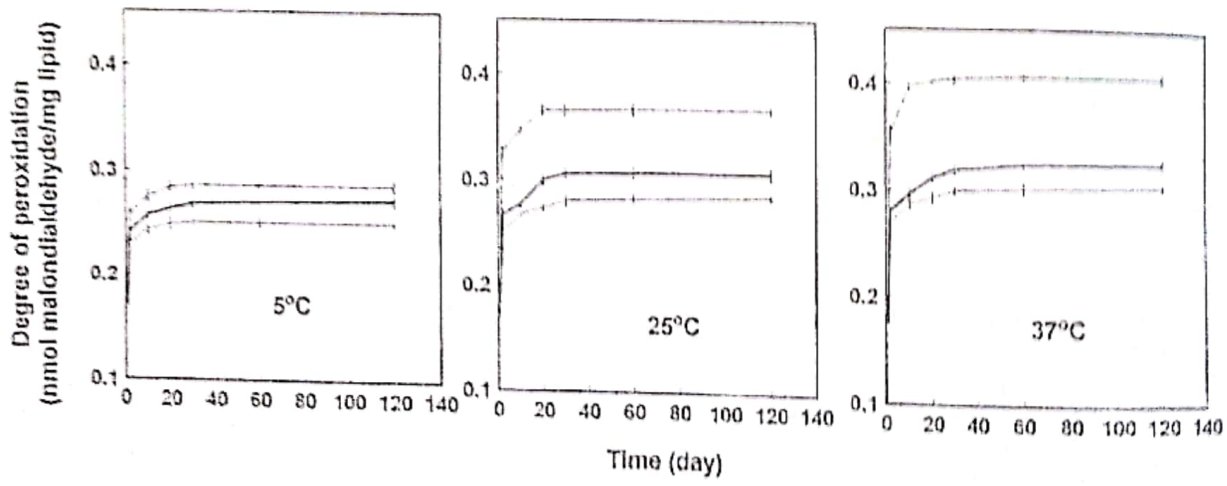

Fig.(3): Time coarse of lipid peroxidation of different multilamellar liposomal formulations consisting of phosphatidylcholine and cholesterol (33 mole %) with various charged lipids (neutral —·— ,positively — ,negatively .....). Liposomes were stored for various times (0,1,5,10,20,30,60 and 120 days) at 5,25,37°C.The vertical bars indicated standard error of the mean (n=3).

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EXPERIMENTAL

1. Materials :

Cholesterol, dicetyl phosphate, stearylamine were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Phosphatidylcholine was prepared from egg yolk of hens following the Faure method [14], and was furthermore purified by using silica gel column . The purity of phosphatidylcholine was checked by thin-layer chromatography on silica gel plates with chloroform-methanol-water (65 : 25 : 4 , by volumes) as solvent system . Chloroform slutions of phosphatidylcholine were stored under nitrogen gas in sealed ampoules at -20° C until use .

Polycarbonate membranes and membrane holders were obtained from Nucleopore Corp. All other reagents and solvents were of a highest grade commercially available .

2. Methods :

2.1. Liposomal preparations :

Two different types of liposomes were prepared, one was prepared by the hand shaking method [15] to produce multilamellar type, and the other was prepared by sonication to produce small unilamellar type [16]. For both unilamellar and multilamellar liposomal types, three different lipid compositions were used to produce neutral, negatively and postively charged liposomes as follows : **a)** neutral liposomes were composed of phosphatidylcholine and the required quantities of cholesterol necessary to obtain the desired molar ratio. **b)** negatively charged liposomes were composed of phosphatidylcholine and dicetyl phosphate in 7: 1 molar ratio with varying amounts of cholesterol. **c)** positively charged liposomes were composed of phosphatidylcholine and stearylamine in 7: 1 molar ratio with varying amounts of cholesterol .

For all formulations (neutral, negatively and positively charged), the cholesterol contents of liposomes were : 0, 10, 20, 30, 33, 35 and 40 mole % (with respect to total lipids) .

Preparation of multilamellar liposomes

Multilamellar liposomes were prepared by the film method which has been described in detail by Bangham et al. [15] . Briefly, the lipid mixtures in chloroform were deposited as a thin film in a round- bottom flask by reduced pressure rotary evaporation . After removal of the final traces of the chloroform with a stream of nitrogen gas and subsequent evacuation, the dried lipid film was hydrated with the appropriate omount of isotonic phosphate buffer solution (pH 7.4±0.2) to give 60 µmol lipid/ml. The resulting suspention was gently shaken for about 1 hour under nitrogen gas at 25°C. Finally, the liposomal lipid concentration was adjusted to 3.6 µmol lipid /ml with the same buffer and filtered through 3.0 µm polycarbonate membrane filter to remove large aggregate of liposomes .

Preparation of small unilamallar liposomes

This type of liposome was prepared from the priviously formed multilamellar liposome by sonication . Multilamellar liposomes were sonicated in a bath-type sonicator under nitrogen gas at 20° C for about 30 minutes. The temperature was regulated using ice-water mixture (60 sconds sonication followed by 30 seconds cooling).

As a result of this procedure multilamellar liposomes were converted to small unilamelar liposomes, and this was indicated by clarification of the turbid suspension[16] .

2.2. Liposomal homogeneity and size destribution

For both types of preparations, the liposomal suspention was forced several times by extrusion through defined pore size of polycarbonate membrane filters. Sequential extrusion was done at a pressure of approximately 5-8 kp/cm^2 in a 25 mm membrane holder . Multilamellar liposomes were sequentially extruded through polycarbonate membranes with pore size of 3.0, 2.0, 1.0 and 0.8 µm . On the other hand, small unilamillar liposomes were sequentially extruded through 0.6, 0.1 and 0.08 µm pore size membrane filters .

The liposomal size of these extrusions could be designated by the final or smallest membrane size through which the suspension was extruded where it was reported in our previous paper[17] and others[18] that a homogeneous preparation with controlled particle size distribution was obtained by this extrusion process .

2.3. Liposomal surface charge

The net surface charge of liposomol preparations was determined by electrophortic mobility . The direction of migration of the liposomol particles was determined by using Carl Zeiss cytopherometer, and this was used to indicat the sign of the net charge prevailing at the liposomal surface.

2.4. Freeze-thawing and freeze- drying procedures

For the freeze-thawing procedure, samples of the final liposomal preparations were frozen in the presence of phosphate buffer (pH 7.4 ± 0.2) containing 7.5% w/v lactose (as cryoprotectant) . Freezing was carried out at - 20°C in glass vials and stored at -20°C until required . After different periods of storage (0, 1, 5, 10, 20, 60 and 120 days) the frozen samples were thawed at room temperature. The suspension was then extruded through polycarbonate membrane filters (as it was stated before) to remove any large aggregate of liposomes .

For the freeze-drying procedure (lyophilizaion), the frozen samples (as described above) were dried under reduced pressure for 12 hours and then stored at - 20°C until required . After the storge periods, samples were rehydrated with distilled water by adding the same quantity losted during lyophilization process and extruded through polycarbonate membrane filters (as it was stated before) .

According to these procedures, two types of liposomes were prepared and could be referred or designated as :

1) Freeze-thawed liposome : which was frozen, stored and then thawed .

2) Freeze-dried liposome : which was lyophilized, stored and then rehydrated .

2.5. Liposomal storage conditions

For choosing the proper storage conditions, to optimize or maintain liposomal stability, the testing protocol was performed under the following conditions :

1- Storage temperature : the liposomal suspensions were stored at 5, 25 and 37°C . Freeze-dried and freeze-thawed liposomes were stored at -20°C.

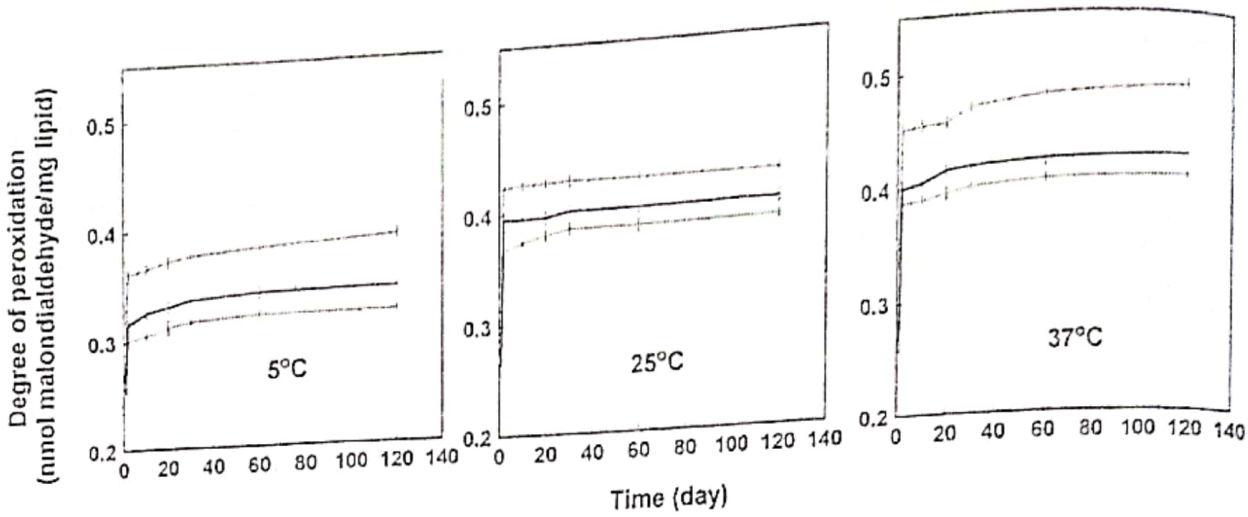

Fig.(4): Time coarse of lipid peroxidation of different small unilamellar liposomal formulations consisting of phosphatidylcholine and cholesterol (33 mole %) with different charged lipids (neutral — -- ,positively — ,negatively .....) Liposomes were stored for various times (0,1,5,10,20,30,60 and 120 days) at 5,25,37°C.The vertical bars indicated standard error of the mean (n=3).

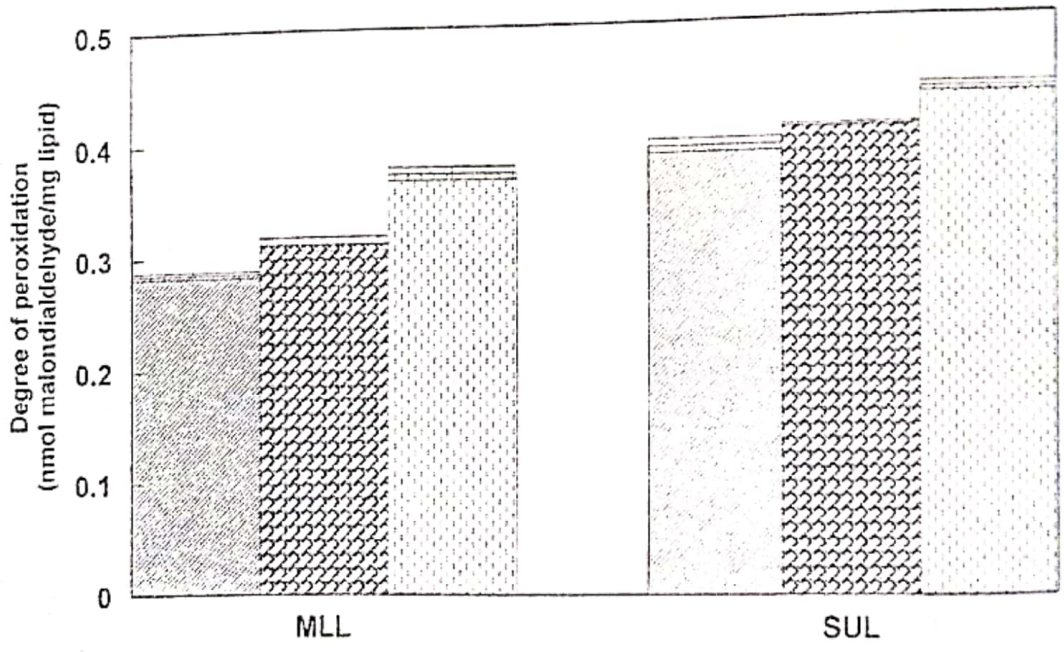

Fig.(5): Degree of lipid peroxidation of multilamellar liposomes (MLL)and small unilamellar liposomes (SUL) consisting of phosphatidylcholine , cholesterol (33 mole %) with various charged lipids (negatively , positively and neutral). Liposomes were stored for one year at 25 °C.The vertical bars indicated standard error of the mean (n=3).

2- Storage time : the liposomal stability was chemically followed during a storage periods of 0, 1, 5, 10, 20, 30, 60 and 120 days. Liposomes which exposed to a temperature of 25°C were stored for 0, 1, 5, 10, 20, 30, 60, 120 and an extended period of 365 days . However, freeze-dried and freeze-thawed liposomes were stored for 0, 1, 5, 10, 20, 60 and 120 days.

It should be pointed out here that, time of measurements which performed immediately after preparation of liposomes or freeze-thawed and freeze-dried liposomes was concerned as zero time and used as control.

2.6. Thiobarbituric acid assay

Liposomal lipid peroxidation, during different storge periods and different storage temperatures, was followed and estimated by using the thiobarbituric acid test [10]. 1.39 ml of liposomal suspension (containing 0.72 mg lipid/ml) were made up to 4.0 ml with distilled water, and were mixed with 1.0 ml of thiobarbituric acid reagent (0.67% thiobarbituric acid aqueous solution and glacial acetic acid, 1 : 1 v/v) . This mixture was heated at 95° C in an oil bath for 1 hour . It is prefererable to prepare the thiobarbituric acid reagent just before use . The reaction product had an absorption peak at 535 nm and was measured using Perkin-Elmer Lamba 3B UV/ VIS spectrophotometer .

The absorbance of sample of liposomal prepartions was measured, against a blank containing the complete assay mixture without liposomes, and expressed in terms of malondialdehyde (nmol / mg lipid). Each experiment was triplicated and the mean values were recorded .

2.7. Statistical Evaluation

Student's t-test was used to compare two groups. However, one-way analysis of variance (ANOVA) was used to test the significance of differences among three groups (neutral, positively and negatively charged liposomes).

RESULTS AND DISCUSSION

The effect of incorporation of various amount of cholesterol into the phospholipid bilayers structure on lipid peroxidation was investigated in the following two liposomal systems : (a) multilamellar liposomes and (b) small unilamellar liposomes. For both types, this study was conducted on neutral, negatively and positively charged liposomes (see Methods) . Measurements were made at 5, 25 and 37°C for freshly prepared suspensions of liposomes (concerned as zero time and used as control) and also after different periods of storage time (1, 5, 10, 20, 30, 60 and 120 days). In all cases of liposomal formulations, it was found that incorporation of cholesterol into other phosolipids, generally, reduced the lipid peroxidation (Figures 1-4) . Also, this effect was most pronounced with 33 mole % cholesterol (as indicated in Figures 1 and 2).

Therfore, these results showed that cholesterol has a suppressive effect on the rate of lipid peroxidation process, during storage, and adjusting its content to 33 mole% provided the appropriate molar ratio for optimal condition which reflects or indicated maximum stability of liposomal system . The orientation and better or optimal packing characteristics of the sterol hydroxyl group (3-ß-OH) at the hydrophilic- hydrophobic interface must be an important factor in the ability of cholesterol to stabilize the liposomal bilayer configuration . It was also postulated that, the mechanisms by which cholesterol stabilize the liposomal phospholipid structure were probably related both to the structural properties of these lipids and to their interactions within liposomal bilayers, where cholesterol has an important modulatory effect on the phospholipid phase changes [5,6].

On the basis of the above results, 33 mole% cholesterol was selected to study the effect of liposomal type, surface charge, temperature and freezing on lipid peroxidation during storage .

Concerning the effect of liposomal types on lipid peroxidation, Figures 3-5 showed that liposomes prepared by sonication (small unilamellar type) provided a greater lipid peroxidation values compared to that prepared by handshaking method of the same charge (multilamellar type) . A comparison between these two types of liposomes, within the same liposomal charge (Figure 5), for the degree of peroxidation (after one year of storage at 25°C) indicated that the statistical differences were significant ($p < 0.05$) . This could be explained on the consideration that, the sonication of liposomes causes oxidation and chemical degradation . It was also reported that the high energy of sonication induced structral and functional changes in the liposomal membranes, which lead to perturbation of the lipid bilayers . This perturbation or disruption of the membrane structure may stimulate the peroxidation process [8].

Also, the effect of liposomal surface charge on lipid peroxidation was studed . The results of this study clearly showed that the degree of peroxidation was variable due to change in liposomal surface charge (as shown in Figures 3-5) . Generally, a comparison between different surface charges, within the same liposomal type, for the degree of peroxidation (after one year of storage at 25°C) indicated that the presence of charged lipid significantly ($P < 0.05$) reduces the lipid peroxidation (Figure 5). Additionally, negatively charged liposomes provided lower peroxidation values as compared to positively charged ones of the same type, however the statistical differences were insignificant ($P < 0.1$) .

Thus, on the basis of these latter observations, it seems possible to propose that inclusion of charged lipid in the liposomal lipid composition results in an structral stabilizing effect of liposomal suspentions . This stabilizing effect of charged lipids could be attributed to the steric barrier on the liposome surface presented by this charge . It was also reported that, these samll vesicles of liposomal system were not thermodynamically stable, and the presence of a charge inducing agent will tend to increase the interlamellar resistance between successive bilayers and thereby providing extra stability against aggregation and fusion [2,19,20].

Regarding the effect of storage temperatures (5, 25 and 37°C) on lipid peroxidation, generally in all types of liposomal formulations, it has been found that the higher the storage temperature the bigger the degree of peroxidation (Figures 3 and 4). Apparently, from the above presented results , it should be emphasized that : 1) The liposomal stability was strongly dependent on the storage temperature . 2) Charged multilamellar liposomes represented an optimal or most suitable formulation for extending the shelf-life and showed higher

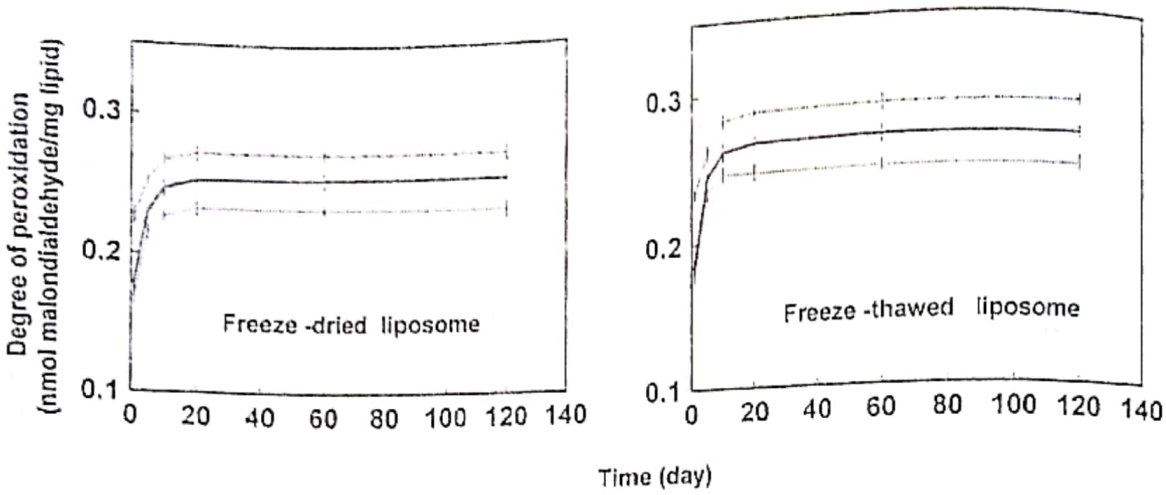

Fig.(6): Time coarse of lipid peroxidation of Freeze-dried and Freeze-thawed multilamellar liposomal formulations consisting of phosphatidylcholine and cholesterol (33 mole %) with different charged lipids (neutral—-- ,positively —, negatively .....). Liposomes were stored for various times (0,1,5,10,20,60 and 120 days) at -20°C. The vertical bars indicated standard error of the mean (n=3).

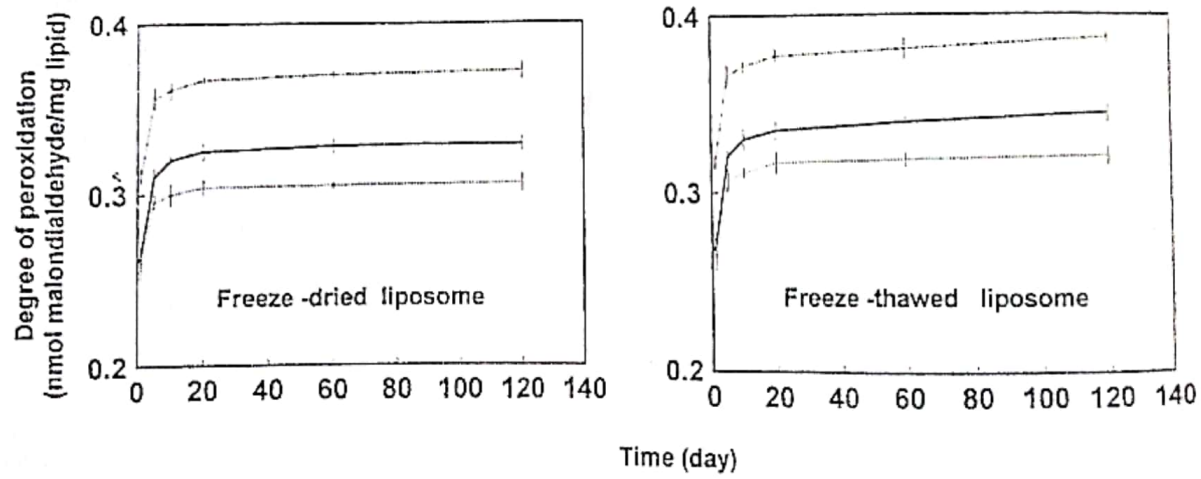

Fig.(7): Time coarse of lipid peroxidation of Freeze-dried and Freeze-thawed small unilamellar liposomal formulations consisting of phosphatidylcholine and cholesterol (33 mole %) with different charged lipids (neutral—-- ,positively —, negatively .....). Liposomes were stored for various times (0,1,5,10,20,60 and 120 days) at -20°C. The vertical bars indicated standard error of the mean (n=3).

stability when stored at low temperature than when stored at high temperature .

Finally, the stability of freeze-dried and freeze-thawed liposomes (see material and methods) was followed during 120 days of storage at -20°C . It was observed that, freeze-dried liposomes proved to be superior to freeze-thawed liposome of the same liposomal type (multilamellar and unilamellar) and of the same charge, in facilitating optimal long term storage conditions, where they showed high stability during 120 days of storage at -20° C (Figures 6-8) . This result could be explained on the consideration that, freeze-thawing causes perturbation in the usual structre of the lipid bilayer, resulting in defects or disorder within the liposomal bilayer configuration, which caused changes in the structural and barrier function [21,22].

Also, a comparison between different surface charges, within the same liposomal type (multilamellar or unilamellar) and also the same storage condition (freeze-thawed or freeze-dried), was made for the degree of peroxidation after 120 days of storage and summarized in Figure 8 . Negatively charged liposomes showed the lowest peroxidation values, and neutral liposomes showed the highest values, as compared to others of the same type and storage condition .

In addition, Figure 8 revealed that small unilamellar liposomes provided a greater peroxidation values compared to multilamellar type of the same charge and storage condition . Consequently, the results of the freezing experiments demonstrated that, freeze-dried negatively charged multilamellar liposomes proved to be the most suitable design, compared to other formulations, for providing the optimal storage conditions and retaining or maintaining the liposomal stability by minimizing peroxidation reactions .

On the basis of the presented results and discusion, it can be concluded that :

1- Cholesterol has a suppressive effect on the rate of lipid peroxidation process, during storage, and adjusting its content to 33 mole% could provided the appropriate molar ration for optimal condition which reflects the maximum stability of liposomal system .

2- Small unilamellar liposomes showed a greater peroxidation values compared to multilamellar type of the same charge and storage condition .

3- Negatively charged liposomes showed the lowest peroxidation values, and neutral liposomes showed the highest values, as compared to others of the same type and storage conditions .

4- The liposomal stability was strongly dependent on the storage temperature, where they showed higher stability when stored at low temperature than when stored at high temperature .

5- Freeze-dried liposomes proved to be superior to freeze-thawed liposomes of the same liposomal type (multilamellar or unilamellar) and of the same charge . Also, negatively charged multilamellar freeze-dried liposomes could provide an optimal storage condition for maintaining liposomal stability .

Accordingly, it should be stressed that liposomal types, lipid composition and surface charge can be modified in a variety of ways and so can potentially be tailored to satisfy the needs of the formulation for solving the stability problems of liposomes . Also, the freezing experiments confirmed the importance of freeze-drying as the appropriate condition or methodology for further optimization of the liposomal system and its proper utilization for useful application, with respect to their stability .

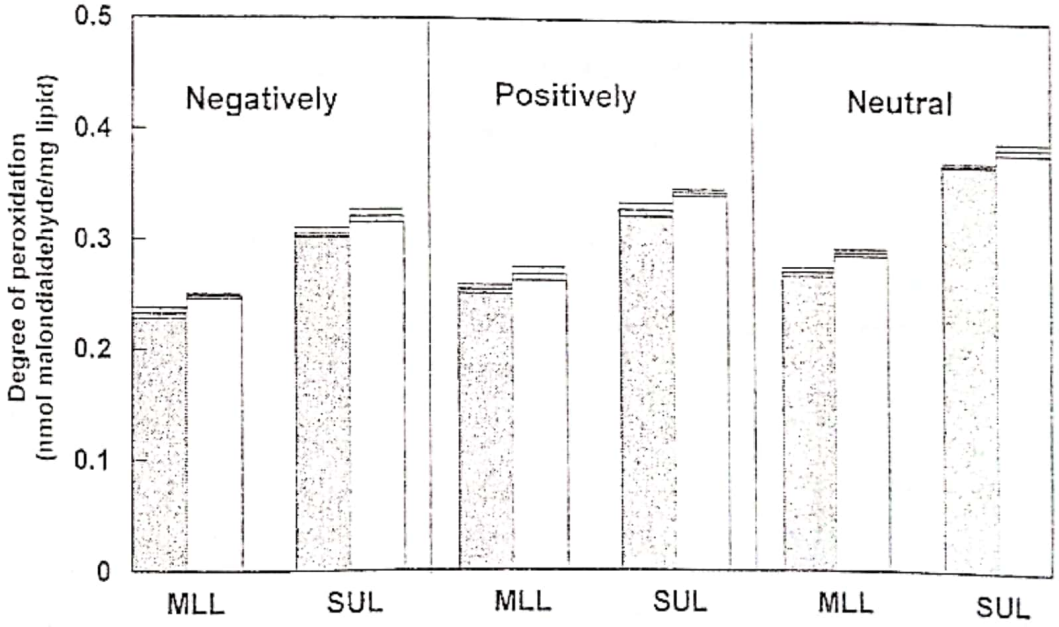

Fig.(8): Degree of lipid peroxidation of freeze-dried ▒ and freeze-thawed □ multilamellar liposomes (MLL)and small unilamellar liposomes (SUL) consisting of phosphatidylcholine and cholesterol (33 mole %) with various charged lipids (negatively,positively and neutral). Liposomes were stored for 120days at -20 °C .The vertical bars indicated standard error of the mean (n=3).

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دراسات على ثبات الليبوزوم بدلالة تركيب الليبيد ، نوع الليبزوم ، الشحنة الموجوده على سطحه وكذلك حالات تخزينـــه

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تم دراسة تأثير تركيب الليبيد ، نوع الليبوزوم ، الشحنة الموجوده على سطحه ، وكذلك حالات التخزين (الزمن ودرجة الحرارة) على بات الليبوزوم . ولقد تم تتبع أكسدة الليبيد فى التركيبات المختلفه لليبوزوم كيميائيا خلال تخزينه تحت ظروف مختلفه باستخدام طريقة حض الثيوباربتيورك للتحليل . حضر الليبوزوم المتعدد الطبقات وكذلك الصغير وحيد الطبقة من اللسيثين المستخرج من صفار البيض مع ضافة كميات مختلفة من الكولستيرول وكذلك مواد تعطى شحنة . ومن أجل ذلك استخدم الداى ستيل فوسفات لاعطاء شحنة سالبه أو ستباريل أمين لاعطاء شحنة موجبة . وأظهرت النتائج أن إضافة الكولستيرول الى تركيب الليبوزوم يقلل عموما من معدل أكسدة الليبيد كذلك فإن ضبط نسبة الكولستيرول المضافة الى ٣٣٪ يعطى الكميه الملائمه لأعلى درجة ثبات لليبوزوم . وبمقارنة أنواع الليبوزوم التى لها فس الشحنة وتحت نفس ظروف التخزين أظهرت النتائج ان الليبوزوم الصغير وحيد الطبقة له درجة أكسدة عالية بالنسبة الى الليبوزوم لمتعدد الطبقات . ومن ناحية الشحنة الموجوده على سطح الليبوزوم فان أقل درجة أكسدة كانت فى الليبوزوم ذو الشحنة السالبة وأعلاها كان ى الليبوزوم المتعادل الشحنة . وبذلك فإن أحتواء الليبوزوم على شحنة كان له تأثير فى ثبات تركيبه وقد يعود ذلك الى الحائل الفراغى لموجود على سطح الليبوزوم بسبب تلك الشحنة . وبالإضافة الى ذلك وبالأخذ فى الاعتبار تأثير درجة حرارة التخزين فى أكسدة الليبيد فإن لليبوزوم أظهر درجة ثبات عاليه عند التخزين فى درجات حرارة منخفضه من تلك التى خزنت عند درجات حرارة عاليه .

وكذلك تم تحضير نوعين من الليبوزوم وهما الليبوزوم المجمد والليبوزوم المجفد وقورنا بالليبوزوم العادى وذلك لاختيار انسب الحالات لملائمه لثبات الليبوزوم عند تخزينه . ووجد أن الليبوزوم المجفد ذو الشحنة السالبة ومتعدد الطبقات يعطى افضل حالات تخزين ملائمة لمحافظة علي ثبات الليبوزوم . ويستخلص من ذلك أن تركيب الليبيد ، نوع الليبوزوم ، الشحنة الموجوده على سطحه وكذلك حالات تخزينه كن التحكم فيها بطرق مختلفه لتؤدى متطلبات صيغاتها فى الصورة المناسبه لحل مشاكل ثبات الليبوزوم .