

EFFECT OF LYOPHILIZATION ON THE CHARACTERISTICS AND STABILITY OF MELANIN LOADED LIPOSOMES

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استهدف هذا البحث دراسة استخدام تقنية التجفيف بالتبريد لزيادة ثبات ليبوزومات (جسيمات شحمية) محملة بمادة ميلانين. تم استخدام نوعين من حاميات التجمد هما لاکتوز وسكروز في تركيزين مختلفين 5% و 10% لكل نوع. وقد أظهرت النتائج تطابق الخصائص الفيزيائية للليبوزومات المحضرة و المجففة بالتبريد مع 10% لاکتوز الطازجة أو المخزونة لمدة سنة في درجة حرارة 5 درجة مئوية مع خصائص الليبوزومات المحضرة حديثاً من حيث درجة الحرارة الانتقالية و التركيب و الشكل و الحجم. كانت درجة الحرارة الانتقالية للليبوزومات المحضرة حديثاً 41.6 درجة مئوية و كان متوسط الحجم 5.21 ميكرومتر. كما بينت النتائج أن الليبوزومات غير المجففة و المخزنة لمدة سنة في درجة حرارة 5 درجة مئوية أظهرت انخفاضاً له دلالة احصائية في درجة الحرارة الانتقالية الى 32.8 درجة مئوية و ازدياد في الحجم الى 15.6 ميكرومتر مع تكوين ليبوزومات متعددة الطبقات كشكل طبقات البصل. و كذلك وجد ان معدل التغير في كمية ميلانين المحمل داخل الليبوزومات قد قل بصورة ذات دلالة احصائية بالتجفيف بالتبريد باستخدام حاميات التجمد المختلفة و خاصة لاکتوز و قد زاد هذا التأثير بصورة ذات دلالة احصائية مع زيادة تركيز حاميات التجمد. كما دلت الدراسة باستخدام جهاز التحليل الطيفي للكتلة أن الميلانين المحمل في الليبوزومات المحضرة و المجففة بالتبريد مع 10% لاکتوز احتفظ بثابته كيميائياً لمدة ستة أشهر عند خمس درجات مئوية و خلاصة البحث أن التجفيف بالتبريد باستخدام 10% لاکتوز يحافظ على ثبات مادة ميلانين كيميائياً و ثبات الليبوزومات المحملة بمادة ميلانين فيزيائياً.

The present study was directed to optimize the stability of melanin liposomes utilizing the technique of lyophilization. Two types of cryoprotectants; sucrose and lactose, each in two concentrations of 5% and 10% were used. Lyophilized liposomes (10% lactose) either fresh or stored for one year at 5°C showed no significant changes ($P>0.05$) in the phase transition temperatures (T_c), structure and shape, and size distribution of the fresh unlyophilized liposomes. The fresh unlyophilized liposomes were unilamellar with T_c of 41.6°C and an average size of 5.21µm. The stored unlyophilized liposomes showed a significant ($P<0.05$) decrease in T_c (32.8°C) and increase in the average size (15.6µm) with the formation of onion-like multilamellar vesicles compared with the fresh unlyophilized ones. Lyophilization of melanin liposomes with different cryoprotectants significantly ($P<0.001$) decreased the rate of leakage of entrapped melanin from the liposomal structure compared with the unlyophilized ones. This cryoprotection effect was significantly ($P<0.05$) increased by the use of lactose and by increasing the cryoprotectant concentration. The entrapped melanin in lyophilized liposomes with 10% lactose was chemically stable for six months at 5°C as evaluated by mass spectroscopic analysis. As a conclusion, lyophilization with 10% lactose maintained the chemical stability of melanin and significantly improved the physical stability of melanin liposomes.

Key words: Melanin; lyophilization; cryoprotectants; stability; liposomes

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Introduction

Freeze drying is a frequently used technique to improve the stability of systems which are unstable in an aqueous environment, such as certain proteins and liposomes (1). Various studies were concerned with the physical stability of liposomes at the time of preparation and upon storage. Liposomes have the tendency to fuse and grow into larger vesicle that is thermodynamically a more favorable state. A more important problem is the breakage of liposomes and drug leakage from the vesicles on storage. It has been reported that liposomes containing drug molecules can be lyophilized and reconstituted with significant drug retention and without significant change in the mean size (1). However, both freezing and drying can induce structural and functional damage to liposomes. Freezing may induce membrane damage from osmotic stress, phase changes, or from freeze-dehydration (2-4). Membrane dehydration during freezing and drying induces lateral stresses that may lead to membrane deformation, lateral phase separations, gel/liquid crystalline phase transitions and lamellar to hexagonal phase transitions (4).

Protection of liposomes against dehydration stress (lyoprotection) can be provided by the addition of sugars which form a solid, amorphous, glassy matrix around the liposomes during freezing and exhibit a low molecular mobility after drying (5). The stability of such dried systems is the subject of a growing number of investigations (6-8). Disaccharides such as sucrose, trehalose, α -lactose and maltose are often used because of their capacity for cryoprotection and dehydroprotection.

Liposomes loaded with Amphotericin B and formed as dry powder inhaler exhibited increase in shelf life after lyophilization using different cryoprotectants (9). The use of sucrose improved the stability of liposomes containing amikacin and 5-fluorouracil (10,11). Ohtake *et al* (12) studied the effect of sugars on the phase behavior of freeze-dried phospholipids- cholesterol mixture using trehalose and the results showed strong evidence for effectiveness of trehalose in stabilizing cholesterol-containing membrane upon lyophilization. Large liposomes containing sodium diclofenac lyophilized using lactose or mannitol as cryoprotectants and stored in the dry form at 5°C showed an increase in their physical stability (13). The use of lactose as cryoprotectant did not influence the properties of

liposomes regarding their size, encapsulation efficiency and release rate. Although mannitol tends to increase the size and encapsulation efficiency, the lipid bilayers are stabilized and became less permeable to the drug (13).

The rate of freezing is considered as a determining parameter in liposomal stability in which slow freezing leads to liposomes of better stability with smaller increase in particle size on storage compared with that formed with quick freezing (14).

The present work was directed to study the effect of lyophilization on the stability of melanin loaded liposomes using two types of cryoprotectants; sucrose and lactose, each in two different concentrations.

Materials and Methods

Materials:

DL- α -Dipalmitoylphosphatidylcholine (DPPC), cholesterol (CHOL), melanin (synthetic, molecular weight 284), N-(2-Hydroxyethyl) piperazine - N'-(2-ethanesulphonic acid) (HEPES) buffer, Sephadex G-75 (50-100 μ m bead size), lactose monohydrate and sucrose were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Triton X-100 was obtained from PARK (Northampton, U.K). All other chemicals were of analytical grade.

Methods:

Preparation and lyophilization of melanin loaded liposomes:

Melanin loaded liposomes formulated with dipalmitoylphosphatidylcholin (DPPC) and cholesterol (CHOL) in a molar ratio of 20:1, respectively were prepared by the conventional thin film hydration method as described by Hashem *et al* (15). Briefly, the lipid phase (150 mg DPPC and 4 mg CHOL) was dissolved in 5 ml chloroform and the solvent was evaporated under nitrogen and in a reduced pressure by a rotary evaporator adjusted at 100 rpm (Heidolph- Elektso, type VV2000, Germany) until a thin film was deposited on the wall of the flask. The aqueous phase was prepared by dissolving 5 mg melanin in 5 ml HEPES buffer (10 mM) adjusted to pH 13 with 1 M sodium hydroxide. The obtained melanin solution was then readjusted to pH 7 with 0.1 M hydrochloric acid. These steps were adopted because melanin is soluble in alkaline medium and still in solution even after neutralization (16). The lipid film was hydrated with 5 ml containing 5 mg

melanin (3.5 mM). The hydration was continued for 30 min while the flask kept rotating at 200 rpm and then the liposomal dispersion was allowed to stand for further two hours at 55°C. Liposomal dispersion was sonicated in ultrasonic water bath (Retsch, Germany) at frequency of 35 KHz for 15 min. The un-entrapped melanin was removed by size exclusion chromatography using gel chromatography column (2.0 cm in diameter and 30cm in height) packed with Sephadex G-75. The liposomal dispersions were eluted with 10 mM HEPES buffer of pH 7. The obtained liposomal fraction was diluted to obtain a total lipid concentration of 7mg/ml.

A volume of 1ml of the liposomal dispersion was diluted with 1ml of one of the following solutions; 5% lactose (L-5), 10% lactose (L-10), 5% sucrose (S-5) or 10% sucrose (S-10). The 2 ml samples were allowed to freeze to -80°C for 24 hr before being attached to the freeze drying system (Labconco Corporation, type Lyph-lock®, 4.5 liter, U.S.A). The samples were then left for about 24 hr in the freeze dryer till a dry cake is formed.

Physical stability of the lyophilized liposomes :

Physical stability of the lyophilized and unlyophilized melanin liposomes was evaluated by studying the change in the percent drug entrapped, phase transition temperature, particle size, and particle size distribution as a function of time. The lyophilized and unlyophilized liposomes were stored at 5°C.

Drug entrapment:

The percent melanin entrapped was determined in the lyophilized (L-5, L-10, S-5 and S-10) and unlyophilized liposomes after storage at 5°C for different time intervals up to 6 months. The selected time intervals were 0.5, 1, 2, 3, 4, 5 and 6 months. The lyophilized liposomes were re-hydrated with distilled water to obtain a liposomal dispersion of 3.5 mg lipid / ml. Liposomes were separated from un-entrapped melanin in the liposomal dispersion by gel permeation chromatography as previously described. An aliquot of the eluted liposomal dispersion was mixed with an equal volume of 1% Triton X-100 in HEPES buffer of pH 7. The surfactant dissolved the liposomes and yielded a clear solution. The concentration of melanin was determined spectrophotometrically at 400 nm (Perkin Elmer, type Lambda EZ 201, USA) using the method described by Watts *et al* (17). Stock

solution of the drug was prepared by dissolving melanin in HEPES buffer (10mM) adjusted to pH 13 with 1 M sodium hydroxide. Then, the pH of the obtained solution was readjusted to pH 7 with 0.1 M hydrochloric acid. Standard concentrations of melanin were obtained by serial dilutions of the stock solution with HEPES buffer of pH 7. The calibration curves were linear over the concentration range of 5- 100 µg/ml and the sensitivity of the method was 1 µg/ml. The percentage of entrapped melanin at different time intervals (t) was calculated by applying the following equation:

$$\% \text{ Drug remaining entrapped} = \frac{\text{Amount of entrapped melanin at time (t)} \times 100}{\text{Amount of melanin entrapped at zero time}}$$

Thermal analysis:

Differential scanning calorimetry (DSC Shimadzu, Japan) was employed for determination of the phase transition temperature (T_c) of the liposomal membrane. In principle, the phase transition temperatures can be observed as changes in heat capacity and measured with DSC as the endotherm peaks (18). The tested sample (1 ml containing 3.5 mg lipid) was subjected to a constant heating rate of 5°C/min at a temperature range of -50 to 100°C and holding temperature of 100°C. An empty pan served as reference for all DSC scans. All manipulations were carried out in dry cabinet and under nitrogen gas environment to minimize adsorption of water by the hygroscopic sample (18).

The change in the phase transition temperature upon storage at 5°C for one year was studied by evaluating T_c for the fresh and stored lyophilized (L- 10) and unlyophilized melanin liposomes. Formula (L-10) was the one that showed the highest drug retention compared with other formulae. The phase transition temperature of the lyophilized samples was determined after re-hydration with distilled water to obtain a liposomal dispersion of 3.5 mg lipid / ml.

Particle size determination:

The examined liposomal dispersion was scanned and imaged using an Image analyzer (Type SAMAICA, Elbek GmbH, Germany) with a Zeiss Axiotron Microscope and an electronic camera with a magnification power of X1000. Full measurement of the size and size distribution was carried out using SAMAICA automatic image contour analysis

system. The change in the size and size distribution upon storage at 5°C for one year was evaluated for the lyophilized (L-10) and unlyophilized melanin liposomes.

Chemical stability of the melanin entrapped in the lyophilized liposomes:

This study was done to test for the chemical stability of melanin entrapped in the lyophilized liposomes (L-10) upon storage at 5°C for 6 months. A sample of melanin solution was subjected to chemical degradation using the method described by Shosuke (19). This was achieved by the addition of 3% KMnO₄ solution to the free melanin solution with vigorous stirring for at least 60 min. and until the purple color of KMnO₄ persisted for at least 10 min. Solid Na₂SO₃ was added to decompose the residual KMnO₄ and the mixture was kept in hot-water bath for 5 min and then centrifuged. The supernatant containing the degraded product was analyzed by mass spectroscopic method (Hewlett Packard 5890, series 11). The resulted spectrum was compared with the spectrum of melanin solution obtained by dissolving the re-hydrated liposomal dispersion in 1% Triton X-100.

Statistical analysis:

The determined parameters in this study were statistically analyzed by one way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparisons test using Graph Pad In Stat tm statistical software (Version 2.04, 1990-1993). Statistical significance was defined at $P < 0.05$.

Results and Discussion

Drug entrapment:

The change in the percent of melanin entrapped in liposomes stored at 5°C by time was studied for lyophilized and unlyophilized samples. Figure 1 illustrates the variation in the percent entrapment of melanin in the lyophilized liposomes with different concentrations of the cryoprotectants (L-5, L-10, S-5 and S-10) and the unlyophilized liposomes as a function of the storage time at 5°C for a period up to 6 months. It was noticed that lyophilized liposomes showed higher protection against melanin leakage in comparison with unlyophilized ones. Sun *et al* (20) found that the leakage of solutes from dry liposomes was correlated linearly with liposome fusion. Physical separation of the dry liposomes by the bulky sugar glass could be an important factor in preve-

ning liposome fusion in the glassy state (21). The dynamic properties of the glassy state depend mainly on the high viscosity, which lowers the mobility of molecules. High concentrations of sugars like lactose and sucrose effectively prevented liposome fusion and aggregation because dry liposomes are sufficiently separated that fusion would hardly occur. Omidfar *et al* (22) found that the presence of lactose and sucrose in the lyophilization procedure gave a significant protection to the drug against leakage from its liposomes.

To investigate the possible pattern of change in the percent melanin entrapped by time, the obtained data was analyzed mathematically according to zero-order, first-order and Higuchi's diffusion equations. The results indicated that the data for all the tested samples was best fitted to first-order kinetics as illustrated by the values of correlation coefficients (r), which ranged from 0.9925±0.004 to 0.9997±0.003. Similarly, Sun *et al* (20) found that the leakage of solutes from dry liposomes followed the law of first-order kinetics and was correlated linearly with liposome fusion.

Protection against drug leakage from the liposomal structure could be considered as a good measure of the physical stability of the formulated liposomes. Thus, the time of 10% drug leakage was considered as the parameter regarding the expiration date of the liposomal formulations. The expiration dates (T₉₀), the release rate constants (K) and the half- life values (T_{1/2}) for the lyophilized (L-5, L-10, S-5 and S-10) and unlyophilized liposomes were calculated according to the first order kinetics and reported in Table 1. Statistical analysis revealed that there were significant differences ($P < 0.001$) between the expiration date (T₉₀) and half- life values of the unlyophilized liposomes and those of the lyophilized liposomes with different cryoprotectants. Also, there were significant differences ($P < 0.05$) between the expiration dates (T₉₀) and half-life values of lyophilized liposomes with different types and concentrations of cryoprotectants (L5, L10, S5 and S10). So the leakage of entrapped melanin was much slower from the lyophilized liposomes compared with the unlyophilized ones. Cryoprotectants highly retarded escaping of entrapped melanin from its liposomes and this effect was directly proportional to their concentrations. Lactose at its high concentration (10%) gave the most effective protection against drug leakage from liposomes as indicated by its high-extended shelf- life (5.17 months).

Table 1. Rate constants (K), half- life values ($T_{1/2}$) and expiration dates (T_{90}) for the change in the percent melanin entrapped in lyophilized and unlyophilized liposomes stored at 5°C in relation to the storage time.

	Rate constant (K) (Month ⁻¹)	Half life ($T_{1/2}$) (Month)	Expiration date (T_{90}) (Month)
Unlyophilized	0.199 ± 0.0004	3.478 ± 0.005	0.7 ± 0.084
Lyophilized (10% lactose)	0.021 ± 0.0006	33.8 ± 0.06 ^a	5.17 ± 0.065 ^a
Lyophilized (5% lactose)	0.025 ± 0.0005	27.9 ± 0.015 ^{a,b}	3.77 ± 0.098 ^{a,b}
Lyophilized (10% sucrose)	0.022 ± 0.0002	31.4 ± 0.312 ^{a,b,c}	4.67 ± 0.082 ^{a,b,c}
Lyophilized (5% sucrose)	0.030 ± 0.0001	22.8 ± 0.059 ^{a,b,c,d}	3.6 ± 0.019 ^{a,b,c,d}

^a significantly different from the unlyophilized at the corresponding parameter ($P < 0.001$).

^b significantly different from the lyophilized with 10 % lactose at the corresponding parameter ($P < 0.05$).

^c significantly different from the lyophilized with 5 % lactose at the corresponding parameter ($P < 0.05$).

^d significantly different from the lyophilized with 10 % sucrose at the corresponding parameter ($P < 0.05$).

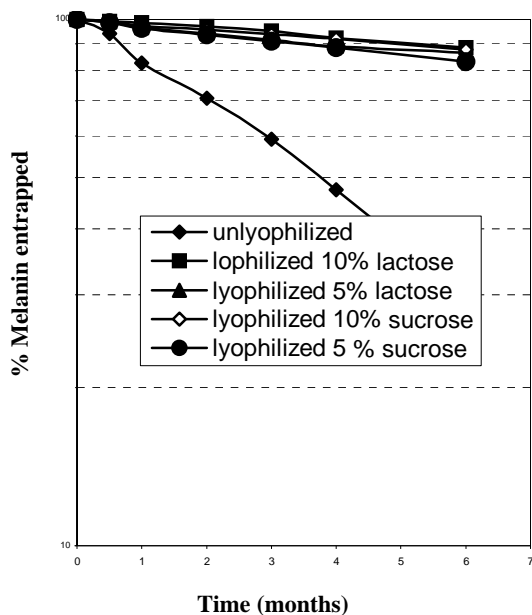


Figure 1. The semi-log plot for the change in the percentage of melanin entrapped in the lyophilized and unlyophilized liposomes stored at 5°C as a function of time.

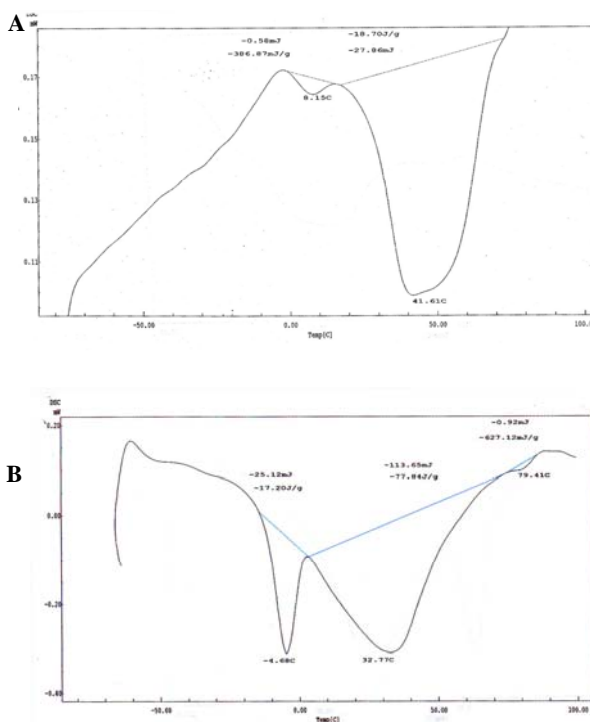


Figure 2. DSC chart with the recorded phase transition temperatures (T_c) for fresh unlyophilized melanin liposomes at 41.61°C (A) and unlyophilized liposomes stored at 5°C for one year at 32.77°C (B).

Thermal analysis:

Figure 2 (A and B) shows the phase transition temperatures recorded for the unlyophilized liposomes freshly prepared and stored at 5°C for one year as measured by the differential scanning calorimetry. Statistical analysis indicated a significant difference ($P < 0.05$) between the phase transition temperatures (T_c) of the freshly prepared (41.6°C) and the stored (32.77°C) unlyophilized liposomes. The phase transition temperature is the minimum temperature required for water to penetrate between the layers of the lipid molecules. The decrease in the phase transition temperature of the unlyophilized liposomes upon storage may be explained by the increase in the hydration level of the liposomal membrane (23). Upon storage of the liposomal dispersion, multilamellar structure was created spontaneously which cause an increase in the hydration level of the liposomal membrane (23).

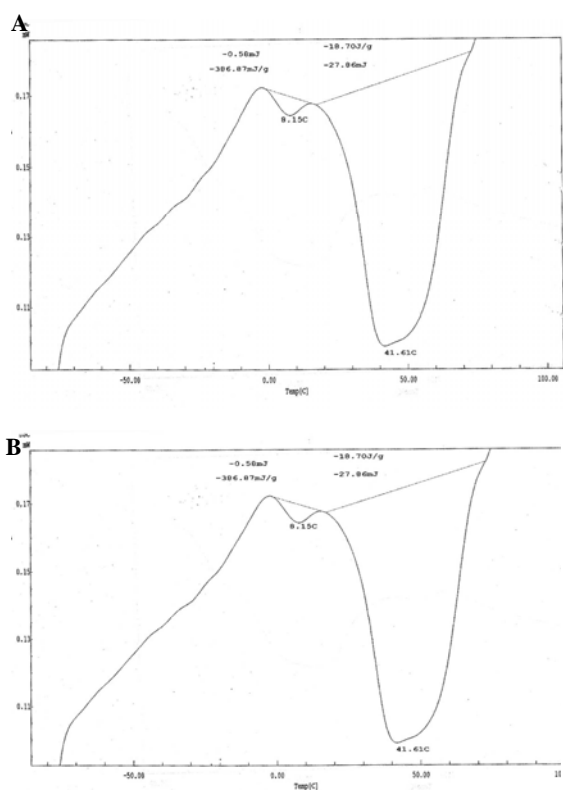


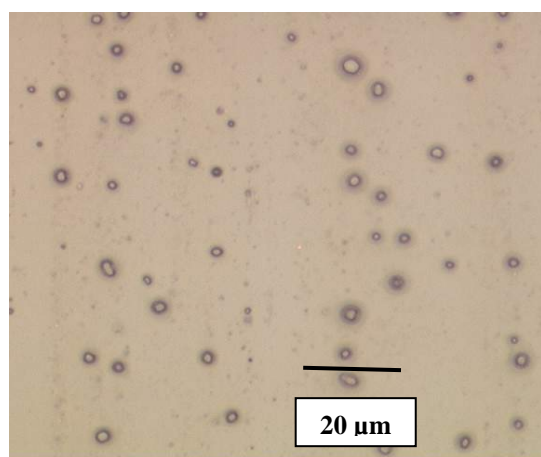
Figure 3. DSC chart with the recorded phase transition temperature (T_c) of 41.61°C for the lyophilized melanin liposomes with 10% lactose both the freshly prepared (A) and the stored at 5°C for one year (B) upon re-hydration.

This result may indicate the necessity of lyophilization to maintain the degree of hydration and consequently the phase transition temperature of the liposomal membrane. Figure 3 shows the typical phase transition temperatures recorded for both the fresh (A) and the stored (B) lyophilized liposomes (L-10). The two types showed a change in the heat capacity at the temperature of 41.61 °C. These results indicated that the use of 10% lactose as a cryoprotectant didn't change the free motion of the molecules forming the liposomal membrane and maintained the phase transition temperature at its value, which is an evidence for stable lyophilized liposomes.

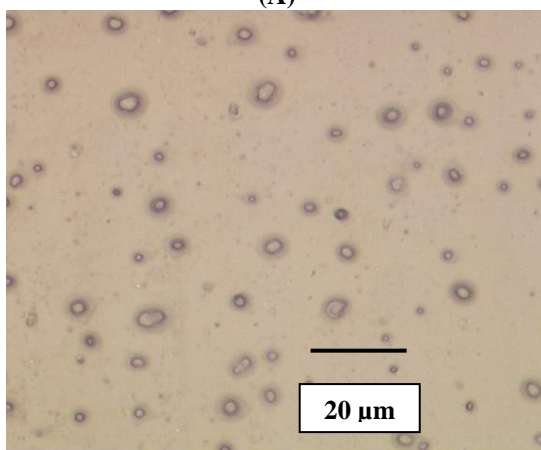
Particle size and particle size distribution:

Figures 4 (A, B, C and D) show photographs for the fresh unlyophilized and lyophilized liposomes, and the stored lyophilized and unlyophilized liposomes at 5°C for one year. There is an enlargement in the size of the stored unlyophilized liposomes with the formation of onion-shape multilamellar vesicles. However, the fresh and stored lyophilized liposomes (L-10) showed no change in the size or shape upon re-hydration. The particle size distribution of the fresh lyophilized liposomes and those stored at 5°C for one year is shown in Figure 5. The most probable size for the fresh lyophilized liposomes was about 6 μm and the average size was $5.31\mu\text{m} \pm 0.09$. The stored lyophilized liposomes had a most probable size of 6 μm and an average size of $5.72\mu\text{m} \pm 0.1$. The fresh unlyophilized liposomes had a most probable size of 6 μm and an average size of $5.21\mu\text{m} \pm 0.09$. Thus, the three samples showed similar particle size distribution. On the other hand, the size distribution of the stored unlyophilized liposomes indicated a most probable size of 16 μm and an average size of $15.6\mu\text{m} \pm 0.1$. Statistical analysis of the size distribution data revealed that, there are no significant differences between the most probable and the average sizes of fresh unlyophilized, fresh lyophilized and stored lyophilized liposomes. However, there was a significant increase ($P < 0.05$) in the size of the unlyophilized liposomes upon storage in comparison with the other liposomal samples. This may be explained by the fusion processes of liposomes upon storage. Williams & Chapman (23) reported that the multilamellar structure is created spontaneously upon storage of the liposomal suspension. These

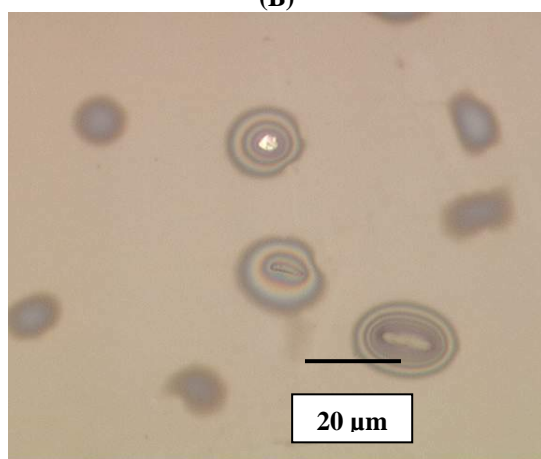
results reflect the significant effect of lyophilization on maintaining the physical stability of the prepared liposomes.



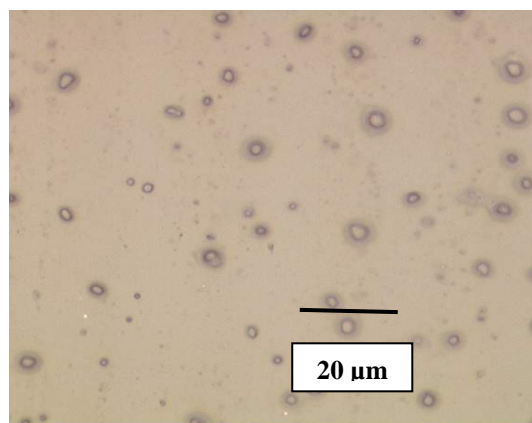
(A)



(B)



(C)



(D)

Figure 4. Photographs of melanin liposomes in HEPES buffer as recorded by Image Analyzer (magnification power X1000).

(A) Fresh unlyophilized liposomes.

(B) Fresh lyophilized liposomes upon re-hydration.

(C) Stored unlyophilized liposomes at 5°C for one year.

(D) Stored lyophilized liposomes at 5°C for one year upon re-hydration.

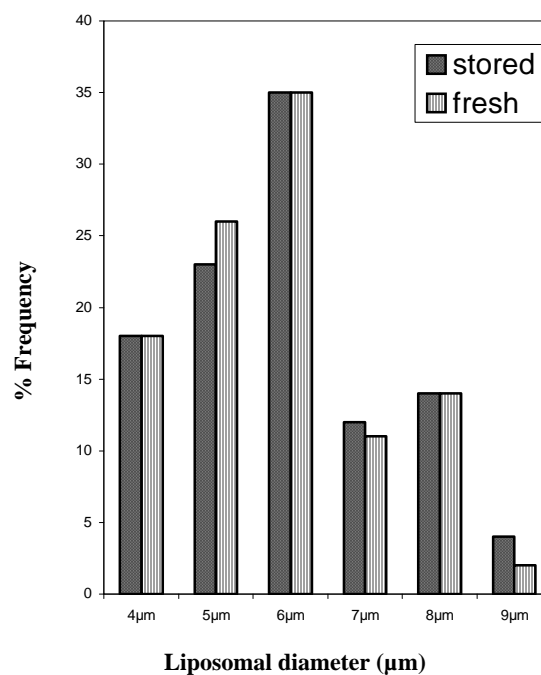


Figure 5. Size distribution of fresh and stored rehydrated lyophilized melanin liposomes formulated with 10% lactose.

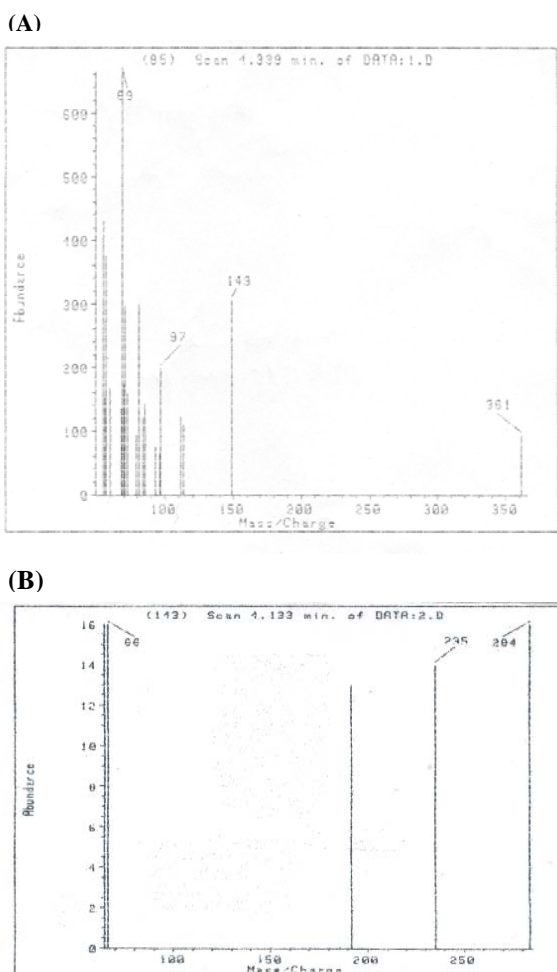


Figure 6. Mass spectra of chemically degraded melanin (A) and melanin solution obtained from lyophilized liposomes with 10% lactose stored at 5°C for 6 months (B).

Chemical stability of melanin liposomes:

Figure 6 (A and B) illustrates the mass spectra of chemically degraded melanin solution and melanin solution obtained from rehydration of the lyophilized liposomes (L-10) stored at 5°C for 6 months. Figure 6 (A) shows different degradation peaks at 143, 97 and 89 m/z. in the spectrum of the degraded melanin whereas; the peak of intact melanin at 284 m/z did not appear. The mass spectrum for lyophilized stored liposomes (B) shows the parent peak of melanin at 284 m/z with no degradation peaks. These results revealed that the process of lyophilization maintained the chemical

stability of entrapped melanin in the lyophilized liposomes (L-10) for a period up to 6 months at 5°C.

Conclusion

The study revealed that lyophilization maintained the physical stability of melanin liposomes with no significant changes in phase transition temperatures, shape and size distribution upon storage at 5°C for up to one year. Also, different cryoprotectants significantly decreased the rate of leakage of entrapped melanin from the liposomal structure compared with the unlyophilized cryoprotectants-free liposomes. Lyophilization with 10% lactose as a cryoprotectant gave the highest protection against liposomal drug leakage. The entrapped melanin in the lyophilized liposomes with 10% lactose was chemically stable upon storage at 5°C for 6 months.

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