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The influence of lipid composition and lamellarity of liposomes on the physical stability of liposomes upon storage

J. du Plessis[a,*], C. Ramachandran[b], N. Weiner[b], D.G. Müller[a]

[a]*Department of Pharmaceutics, Potchefstroom University for CHE, Potchefstroom 2520, South Africa*
[b]*College of Pharmacy, University of Michigan, Michigan, MI 48109, USA*

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Abstract

The physical stability of the four liposomal systems was determined on storage at 4 and 25°C over a 6-month period. A correlation of the mean volume diameter, zeta potential and pH lead to the conclusion that stability follows the order of egg lecithin (PC)/cholesterol (CH)/stearylamine (SA) < PC/CH/phosphatidylserine (PS) < bovine brain ceramides (CM)/CH/palmitic acid (PA)/CS < PC/CH/cholesteryl sulphate (CS) at 4°C, as well as at 25°C, after a 6-month storage period. Large unilamellar vesicles (REV) proved to be superior to multilamellar liposomes (MLV) and dehydration/rehydration liposomes (DRV) systems as far as physical stability was concerned. Instability was exaggerated in the systems stored at 25°C as compared to storage at 4°C.

Keywords: Physical stability; Liposomes; Lamellarity; Lipid composition; Storage

1. Introduction

Many studies have been performed on the stability of liposomes as a function of time in their freeze-dried form or in solution (Gregoriadis and Davis, 1979; Crommelin and Van Bommel, 1984; Frokjaer et al., 1984; Crommelin et al., 1986; Fransen et al., 1986; Hernández-Caselles et al., 1990). No study, however, has attempted to use the mean volume diameter, zeta potential and pH as parameters for liposomal stability under conditions of compositional changes and at different storage temperatures.

Because it was suspected that the size of the vesicles might be important to their stability, care was taken to measure the mean volume diameter. The diameter of these vesicles per se is not that important, but rather the entrapped volume. Because the volume increases as the cube of the radius, while surface area increases as its square, measurement of the diameter can provide a deceptive estimate of the trapped volume. Based on measurements of the diameter of the vesicles, the average size would appear to be small, but since a small number of vesicles contain a large volume, the volume distribution is skewed towards the larger sizes. We preferred to use the mean volume diameter as an indicator of stability of the liposomes.

* Corresponding author.

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Zeta potential determines the stability of any colloid. A stable colloid is one in which the colloid particles remain separate and distinctly dispersed. An unstable colloid is characterized by particles which gradually agglomerate. If the charge on the particles is high, the particles repel one another and the colloid is stable. If the charge is near zero, the Brownian movement of the particles causes them to collide and to become attached to one another. It is desirable to maximize the particle charge in order to achieve greatest stability. According to the users' manual of the Zeta meter (Pen Kem, Inc., Bedford Hills, NY), a zeta potential of more than 61 mV usually indicates excellent stability, 41–60 mV good stability, 31–40 mV moderate stability and 10–30 mV incipient instability.

Liposomes of four different compositions were prepared for the purpose of this study. Cholesterol was included in the liposomes because it has three recognized effects: (i) increasing the fluidity or microviscosity of the bilayer; (ii) reducing the permeability of the membrane to water soluble molecules; and (iii) stabilizing the membrane in the presence of biological fluids (Weiner et al., 1989).

The presence of negatively charged lipids such as phosphatidylserine (PS) and cholesterylsulphate (CS), or positively charged detergents such as stearylamine (SA), will tend to increase the interlamellar resistance between successive bilayers, and thus lead to a greater overall entrapped volume. The presence of charged lipids also reduces the likelihood of aggregation after preparation of the liposomes.

Skin lipid liposomes consisting of bovine brain ceramides (CM), palmitic acid (PA), cholesterol (CH) and cholesteryl sulphate (CS) were also prepared, because both phospholipid liposomes and skin lipid liposomes are readily used. These lipids are those which could be extracted from the stratum corneum and therefore liposomes with a similar composition, using synthetic chemicals, were prepared. The formulation of stable liposomes from mixtures of skin lipids has been documented by Weiner et al. (1989). The stratum corneum of humans, mice and pigs have been shown to be devoid of phospholipids. The lipid composition is non-polar in nature and consists primarily of ceramides (40%), cholesterol (25%), fatty acids (25%) and cholesteryl sulphate (10%).

The objectives of this study were to determine the influence of liposomal composition and preparation method on the physical stability of the liposomes during a 6-month storage period at 4°C and at room temperature (25°C). Mean volume diameter, zeta potential and pH were the parameters used for the determination of the stability of the liposomes.

2. Materials and methods

2.1. Materials

Cholesterol (CH), cholesteryl sulphate (CS), bovine brain ceramides (CM), palmitic acid (PA), stearylamine (SA) and Hepes free acid were obtained from Sigma (St. Louis, MO). Hepes free acid is (*N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid]) and has a buffer capacity of 6.8–8.2 and a pK_a of 7.5 at 25°C. Egg lecithin (PC) was obtained from Avanti Polar Lipids (Birmingham, AL) and α-tocopherol (α-T) from Eastman Kodak (Rochester, NY). All other chemicals were of analytical grade and the water used was double distilled, deionized and filtered with a Milli-Q system (Millipore Corp. Bedford, MA).

2.1.1. Experimental design

The effect of lipid composition was tested by the preparation of liposomes containing PC/CH/SA, PC/CH/PS and PC/CH/CS, all at molar ratios of 1:0.5:0.1 and CM/CH/PA/CS at a weight ratio of 4:2.5:2.5:1. The liposomes were prepared to have final total lipid concentration of 50 mg/ml. One percent of alpha-tocopherol (an antioxidant) was added to the lipid phase of all liposomes. Each of these formulations was prepared according to three different preparation methods to obtain different types of liposomes, i.e., multilamellar (MLV), large unilamellar vesicles (LUV) and dehydration-rehydration (DRV) liposomes.

2.2. Methods

2.2.1. Preparation of liposomes

2.2.1.1. Multilamellar liposomes (MLV). Multilamellar liposomes (MLV) were prepared, using the conventional film method (Bangham et al., 1965). The lipid mixtures were dissolved in a 2:1 (v/v) mixture of chloroform and methanol and deposited as a thin film in a round-bottom flask by rotary evaporation under nitrogen at 37°C. The flask containing the lipid film was stored overnight under vacuum to facilitate removal of residual solvents. The films were hydrated for about 40 min by the addition of an isotonic 0.05 M Hepes buffer, pH 7.4, with mild agitation at 45°C. The final concentrations of lipid was 50 mg/ml. The liposomal preparations were examined with a Nikon Diaphot Light microscope to ensure liposomal quality and integrity and to confirm their lamellarity.

2.2.1.2. Large unilamellar vesicles (REV). Large unilamellar vesicles were prepared by a modification of the reverse-phase evaporation method (REV) of Szoka and Papahadjopoulos (1978). Appropriate amounts of the lipid mixtures were dissolved in 10 ml of a chloroform-methanol mixture (2:1; v/v). Five ml of 0.05 M Hepes buffer (pH 7.4) and enough additional methanol (up to 1.5 ml) were added to yield a clear solution after brief sonication. The organic solvents and a small amount of water were then removed under nitrogen at 45°C. Solvent removal was continued until all foaming ceased.

2.2.1.3. Dehydration/rehydration liposomes (DRV). Dehydration/rehydration liposomes (DRV) were prepared by a modification of the method reported by Kirby and Gregoriadis (1984). Appropriate amounts of the various lipids were dissolved in chloroform/methanol (2:1; v/v) in a round-bottomed flask, the solvents were removed using a roto-evaporator under vacuum at 40°C. The flask containing the film was dried overnight in a desiccator to remove residual solvent. An appropriate aliquot of 0.05 M Hepes buffer was then added and the mixture was hydrated at 45°C for about 40 min. Intermittent vortexing was required for complete hydration. The resultant dispersion was then dehydrated at 50°C under vacuum, using the roto-evaporator. When the liposomal suspension became very viscous, an amount of water, equivalent to that removed, was reintroduced into the viscous suspension. The rehydrated liposomes were allowed to equilibrate for about 45 min at 45°C.

2.2.1.4. Determination of the physical stability of liposomes. Liposomal size, zeta potential and pH of the liposome suspensions were the parameters chosen to indicate the physical stability of liposomes. Measurements were performed immediately after preparation of the liposomes and monthly thereafter for a 6-month period. Liposomes were stored in glass vials at 4 and at 25°C for 6 months. The mean volume diameter of the vesicles was determined using a Nicomp Model 370 Submicron Particle sizer (Pacific Scientific Instrument Division, Silver Spring, MD). Zeta potential was determined using a Lazer Zee Model 500 (Pen Kem, Inc., Bedford Hills, NY). The liposomal system was diluted about 100 times with double-distilled water and placed in an electrophoresis chamber consisting of two electrode compartments and a connecting chamber. A voltage of 150 V was applied between the two electrodes, one located in each compartment.

3. Results

The mean volume diameter (μm), zeta potential (mV) and pH of the four different liposomal compositions prepared as MLV, DRV and REV, directly after preparation and after 6 months' storage at 4 and 25°C are shown in Tables 1–3, respectively.

4. Discussion

4.1. Mean volume diameter

A significant difference was found between the physical stability of the four formulations that

was investigated. The positively charged system (PC/CH/SA) showed the largest increase in mean volume diameter as compared to the other three systems both after storage at 4 and at 25°C for 6 months (Table 1). Increases in mean volume diameter of up to 309 and 786% were found at MLV systems after storage at 4°C and for REV systems after storage at 25°C respectively. Additionally, this system also proved to be very irritating to the skin. The combined unfavourable physical stability and the skin irritation thus disqualified this system as a formulation of choice. As expected, a growth in mean volume diameter (Table 1) coincided with a decrease in the zeta potential (Table 2) in most instances.

4.2. Zeta potential

The results of the zeta potential measurements (Table 2) indicate that PC/CH/SA was the most unstable of the four systems investigated. Generally, these results confirmed the importance of a storage temperature of 4°C, however, some discrepancies occurred. It should be stressed that zeta potential is not considered as the most important indicator of liposomal stability unless the measurements can be done in a controlled environment with electronic equipment. One should not only rely on zeta potential measurements, but should always use this in conjunction with mean volume diameter measurements.

4.3. pH

The pH (Table 3) of the skin lipid liposomes was the lowest of all the systems tested. This could be expected, since it contained phosphatidic acid which would lower the pH of the system. Although some changes in pH occurred, no pH lower than 6.4 was found, not even after storage for 6 months at 25°C. Once again, the most pronounced changes took place in the PC/CH/SA system (up to 18% at 25°C). No changes in excess of 10% took place in any of the other three systems which were tested. The lowering in pH might be attributed to chemical instability (oxidation). This, however, is doubtful due to the inclusion of an anti-oxidant in all the systems (Fukuzawa et al., 1981).

4.4. Liposomal composition and lamellarity

The PC/CH/CS system proved to be the most stable when judged according to mean volume diameter, zeta potential and pH. REV proved to be the most stable systems (of all the compositions tested) when physical stability was measured according to the mean volume diameter (Table 1) as well as pH (Table 3). However, sometimes some unexpected low zeta potential values were observed (Table 2). MLV and DRV with compositions PC/CH/SA and CM/CH/PA/CS showed huge increases in mean volume diameter after 6 months' storage (Table 1) (up to 730% for PC/CH/SA and 199% for CM/CH/PA/CS). However, the skin lipid liposomal system showed the best physical stability when measured according to zeta potential (Table 2). At the end of the 6-month testing period, MLV showed the largest increase (up to 650%) in mean volume diameter (Table 1). A possible explanation is that the multilamellar bilayers have fused to form unilamellar vesicles with much larger mean volume diameters.

4.5. Storage conditions

Physical instability in systems were enhanced when systems were stored at 25°C. An increase in storage temperature thus contributed to instability in the systems. Therefore, it is recommended that liposomal systems should be kept refrigerated to achieve the best physical stability.

5. Conclusions

A correlation of particle size, zeta potential and pH data leads to the conclusion that the physical stability of the four liposomal systems which were tested, follows the order of PC/CH/SA < PC/CH/PS < CM/CH/PA/CS < PC/CH/CS at 4°C, as well as at 25°C, after a 6-month storage period. REV proved to be superior to MLV and DRV systems as far as physical stability was concerned.

Table 1
Mean volume diameter (μm) of liposomes of four different compositions prepared as MLV, DRV and REV at 0 h and after 6 months storage at 4 and 25°C respectively (n = 4)

Time and storage conditions	Composition and preparation method of liposomes											
	PC/CH/SA			PC/CH/CS			PC/CH/PS			CM/CH/PA/CS		
	MLV	DRV	REV	MLV	DRV	REV	MLV	DRV	REV	MLV	DRV	REV
0 h	2.51 ± 0.93	2.27 ± 0.50	0.44 ± 0.00	1.90 ± 0.11	2.46 ± 0.08	0.29 ± 0.00	2.06 ± 0.09	1.57 ± 0.06	0.68 ± 0.02	2.57 ± 0.99	2.67 ± 0.26	0.67 ± 0.1
6 months (4°C)	7.78 ± 0.11	3.71 ± 0.09	0.49 ± 0.09	2.57 ± 0.11	2.22 ± 0.06	0.29 ± 0.01	2.91 ± 0.19	1.79 ± 0.02	0.64 ± 0.01	5.58 ± 0.03	2.91 ± 0.02	0.6 ± 0.03
6 months (25°C)	16.36 ± 1.34	16.6 ± 0.98	3.46 ± 0.25	2.42 ± 0.21	2.61 ± 0.53	0.29 ± 0.01	2.49 ± 0.04	1.89 ± 0.02	0.67 ± 0.05	5.13 ± 0.52	4.53 ± 0.44	0.77 ± 0.02

Table 2
eZeta potential (mV) of liposomes of four different compositions prepared as MLV, DRV and REV at 0 h and after 6 months storage at 4 and 25°C respectively (n = 9)

Time and storage conditions	Composition and preparation method of liposomes											
	PC/CH/SA			PC/CH/CS			PC/CH/PS			CM/CH/PA/CS		
	MLV	DRV	REV	MLV	DRV	REV	MLV	DRV	REV	MLV	DRV	REV
0 h	75 ± 4.0	75 ± 3.2	91 ± 3.4	83 ± 5.3	88 ± 1.0	88 ± 3.2	89 ± 6.7	99 ± 5.6	98 ± 5.5	83 ± 3.83	83 ± 4.0	84 ± 6.6
6 months (4°C)	41 ± 2.1	53 ± 4.2	52 ± 1.1	66 ± 0.8	75 ± 1.9	78 ± 1.3	71 ± 0.2	56 ± 1.1	15 ± 2.4	82 ± 1.8	64 ± 2.3	73 ± 3.5
6 months (25°C)	60 ± 1.9	61 ± 2.3	18 ± 0.5	53 ± 1.2	54 ± 0.6	56 ± 0.8	38 ± 0.7	55 ± 2.7	62 ± 2.4	75 ± 2.5	71 ± 1.2	75 ± 1.4

Table 3
pH[a] of liposomes of four different compositions prepared as MLV, DRV and REV at 0 h and after 6 months storage at 4 and 25°C respectively (n = 3)

Time and storage conditions	Composition and preparation method of liposomes											
	PC/CH/SA			PC/CH/CS			PC/CH/PS			CM/CH/PA/CS		
	MLV	DRV	REV	MLV	DRV	REV	MLV	DRV	REV	MLV	DRV	REV
0 h	7.80	7.86	7.88	7.36	7.39	7.36	7.31	7.2	7.33	6.87	6.62	6.53
6 months (4°C)	7.03	7.00	7.54	7.28	7.29	7.24	6.85	6.86	6.9	6.72	6.71	6.55
6 months (25°C)	6.86	6.85	6.47	7.25	7.11	7.25	6.75	6.86	6.95	6.52	6.46	6.55

[a]Standard deviation smaller than 0.01.

References

Bangham, A.D., Standish, M.M. and Watkins, J.C., The action of steroids and streolysins on the permeability of phospholipid structures to cations. *J. Mol. Biol.*, 13 (1965) 138–145.

Crommelin, D.J.A. and Van Bommel, E.M.G., Stability of liposomes on storage: freeze-dried, frozen or as an aqueous dispersion. *Pharm. Res.*, 1 (1984) 159–164.

Crommelin, D.J.A., Fransen, G.J. and Salemink, P.J.M., Stability of liposomes on storage. In: Gregoriadis, G., Senior, J. and Poste, G. (Eds.), *Proceedings of a NATO ASI on Targeting of Drugs with Synthetic Systems*, Plenum Press, New York, 1986, pp. 277–286.

Fransen, C.J., Salemink, P.J.M. and Crommelin, D.J.A., Critical parameters in freezing of liposomes. *Int. J. Pharm.*, 33 (1986) 27–35.

Frokjaer, S., Hjorth, E.L. and Worts, O., Stability testing of liposomes during storage. In: Gregoriadis, G. (Ed.), *Liposomes Technology*, Vol 1, CRC Press, Boca Raton, Florida, 1984, pp. 235–245.

Fukuzawa, K., Chida, H., Tokumura, A. and Tsukatani, H., Antioxidative effect of alpha-tocopherol incorporation into lecithin liposomes on ascorbic-acid-Fe^{2+} induced lipid peroxidation. *Arch. Biochem. Biophys.*, 206 (1981) 173–180.

Gregoriadis, G. and Davis, C., Stability of liposomes in vivo and in vitro is promoted by their cholesterol content and the presence of blood cells. *Biochem. Biophys. Res. Commun.*, 89 (1979) 1287–1293.

Hernández-Caselles, T., Villalaín, J. and Gòmez-Fernàndez, J.C., Stability of liposomes on long term storage. *J. Pharm. Pharmacol.*, 42 (1990) 397–400.

Kirby, C.J. and Gregoriadis, G., A simple procedure for preparing liposomes capable of high encapsulation efficiency under mild conditions. In: Gregoriadis, G. (Ed.), *Liposome Technology*, Vol. 1, CRC Press, Inc., Boca Raton. FL, 1984, pp. 19–28.

Szoka, F. and Papahadjopoulos, D., Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation. *Proc. Natl. Acad. Sci. USA*, 75 (1978) 4191–4198.

Weiner, N., Martin, F. and Riaz, M., Liposomes as a drug delivery system. *Drug Dev. Ind. Pharm.*, 15 (1989) 1523–1554.