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Stability of Liposomes on Long Term Storage

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Abstract—The effect of the lipid composition of liposomes on their storage for up to one year under different environmental conditions has been examined using 5,6-carboxyfluorescein as a model drug. When cholesterol and/or α-tocopherol were included in the liposomes, a significantly greater amount of dye was retained. The presence of α-tocopherol decreased the breakdown of phosphatidylcholine to lysophosphatidylcholine and also reduced the level of peroxidation. Carboxyfluorescein retention was further enhanced when liposomes were stored at 4°C or at room temperature (20°C) in an O_2-free atmosphere. Lysophosphatidylcholine formation also slowed when the liposomes were kept at 4°C, or in an O_2-free atmosphere. It is concluded that egg yolk lecithin liposomes may be stored for long periods at low temperature in an O_2-free atmosphere or with added stabilizers such as cholesterol and α-tocopherol.

Liposomes encapsulating drugs should not leak drug on storage. Although studies have been made on the stability of liposomes with time, when they were either freeze-dried or in solution (Gregoriadis & Davis 1979; Kirby et al 1980; Senior & Gregoriadis 1982; Crommelin & Van Bommel 1984; Crommelin et al 1986; Fransen et al 1986), no study has attempted to relate leakage of liposome-encapsulated compounds with composition changes of liposomes on long term storage. We have used multilamellar liposomes in such a study because they are easy to prepare and are the most obvious candidate for use as a pharmaceutical dosage form.

Materials and Methods

Egg yolk lecithin and α-tocopherol were obtained from Sigma Co., Poole, UK. Cholesterol (Merck, Darmstadt, FRG) was recrystallized twice from methanol, and 5,6-carboxyfluorescein (Eastman Kodak Co., New York, USA) was purified according to Ralston et al (1981). All other chemicals were of analytical grade. Water was twice distilled in an all-glass apparatus and deionized in a Milli-Q apparatus (Millipore).

Liposomes were made under aseptic conditions. Standard solutions of lipids were stored as chloroform–methanol solutions at −40°C and samples taken for preparation as required. Lipid mixtures were prepared by combining the appropriate amounts of lipids in a glass tube the solvent being evaporated under an O_2-free N_2 stream, and the last traces eliminated by desiccation under vacuum for at least 3 h. After the addition of sterile MOPS (3-[*N*-morpholine]-propane sulphonic acid)-buffered saline solution A (5 mM MOPS, 150 mM NaCl, 1 mM EDTA, 0·02% NaN_3, pH 7·4) to give a lipid concentration of 10 mg mL^{-1}, multilamellar liposomes (MLV) were formed by careful mixing in a bench vibrator at room temperature (20°C). Mixing was continued until a homogeneous and uniform suspension was obtained which was then diluted with sterile buffer to give a final lipid concentration of 5 mg mL^{-1}.

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Liposomes without cholesterol were divided into three groups and placed in sterile glass ampoules which were flame-sealed and stored in the dark. Two groups, including one bubbled with O_2-free N_2 for 90 s, were stored at room temperature; the third group was stored at 4°C.

Multilamellar liposomes containing encapsulated 5,6-carboxyfluorescein (CF) were formed following the same protocol, but using 100 mM CF in sterile MOPS-buffered solution B (5 mM MOPS, 50 mM NaCl, 1 mM EDTA, 0·02% NaN_3). To separate encapsulated CF from free CF, liposomes were applied to a sterile Sephadex G-50 Fine column equilibrated with sterile MOPS buffered saline solution A. Eluted liposomes were diluted to 8 times the initial volume; 200 μL amounts were stored in sterile glass ampoules and treated as above.

Lipid phosphorus was assayed by the method of Bartlett (1958). Lipid peroxidation was measured as thiobarbituric acid reactivity (Kunimoto et al 1981) using as standard a freshly prepared solution of malonaldehyde-bis (dimethylacetal). Lipid extraction was as described by Bligh & Dyer (1959).

Fatty acids were determined by gas chromatography using heptadecanoic methylester as standard. Lysophosphatidylcholine was determined as lipid phosphorus after separation from phosphatidylcholine by thin layer chromatography on a silica gel 60 F254 plate (Merck, Darmstadt).

To ascertain if some products of lipid peroxidation could be isolated at the same time as lysophosphatidylcholine, a fresh sample of egg yolk lecithin liposomes was peroxidized with ascorbic acid and $FeSO_4$ as described by Fukuzawa et al (1981). After 1 h of incubation, lipid peroxidation was measured as before. Peroxide content in the peroxidized samples was similar to that found in stored egg yolk lecithin liposomes after 8 days at room temperature in the presence of O_2. Lipid was extracted in the peroxidized and in the control non-peroxidized samples by the method of Bligh & Dyer (1959) and chromatographed on a silicagel 60 F254 plate as before. Lysophosphatidylcholine content was assayed and no differences were found between the two samples.

To measure the CF released, amounts were mixed with 3 mL of MOPS buffered saline solution A and fluorescence was measured at room temperature before (F_i) and after (F_t)

the addition of 100 μL of 10% Triton X-100 (w/v) using a Shimadzu RF-540 spectrofluorophotometer (excitation 492 nm, emission 518 nm). CF retention was calculated from the expression

$$\% \text{ CF retained} = 100 - [((F_i - F_o)100)/(F_t - F_o)]$$

in which F_o was the initial fluorescence of the liposome solution immediately after liposome preparation.

Results

The CF retained in different vesicles consisting of egg yolk lecithin, cholesterol and α-tocopherol is shown as a function of time for different experimental conditions in Fig. 1. Liposomes consisting of egg yolk/lecithin alone were the least retentive at all the conditions tested. The time necessary to observe a 50% decrease in the CF retained ($t_{\frac{1}{2}}$) for egg yolk lecithin liposomes at room temperature was 13 days (Table 1) whereas those at room temperature in O_2-free atmosphere and those at 4°C in the presence of O_2 had $t_{\frac{1}{2}}$ values of 30 and 100 days, respectively (data calculated from Fig. 1). When cholesterol was included in the liposomes, the $t_{\frac{1}{2}}$ at room temperature increased from 13 to 50 days whereas the $t_{\frac{1}{2}}$ at 4°C increased from 100 to 180 days (Table 1). A significant increase in CF retention was also obtained when α-tocopherol was included in liposomes (Fig. 1), either in the absence or presence of cholesterol; α-tocopherol and cholesterol together seemed to act synergistically, at room temperature, the $t_{\frac{1}{2}}$ increasing significantly. The most dramatic effect on CF retention was obtained with liposomes containing α-tocopherol at 4°C (Fig. 1C). Approximately 75% of the CF

Table 1. Time (days) necessary to observe 50% of carboxyfluorescein release ($t_{\frac{1}{2}}$).

Liposomes	Room temp.	Room temp. O_2-free	4°C
EYL[a]	13	30	100
EYL:Chol, 5:1[b]	50	—	180
EYL:α-T, 20:1[b]	100	140	>400
EYL:α-T, 5:1[b]	120	140	>400
EYL:Chol:α-T, 5:1:0·3[b]	190	—	>400

[a] EYL, egg yolk lecithin; Chol, cholesterol; α-T, α-tocopherol.
[b] Figures represent molar ratios.

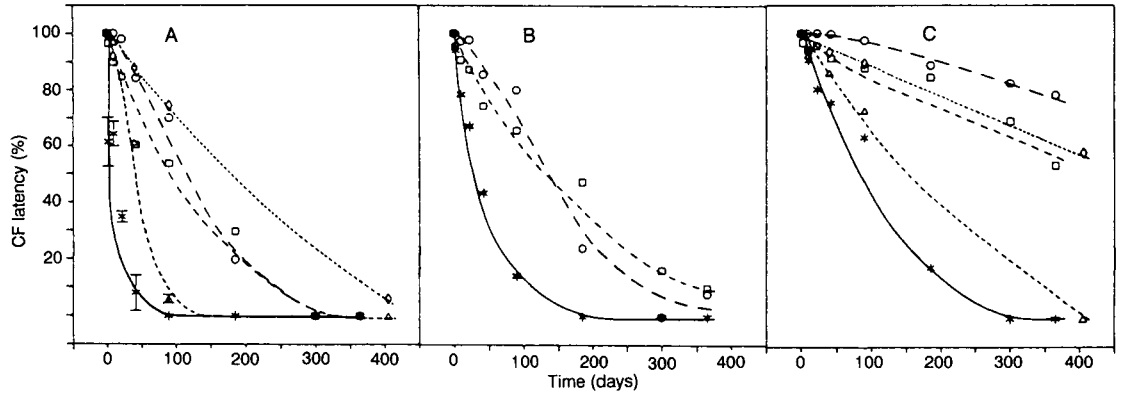

FIG. 1. 5,6-Carboxyfluorescein retention in multilamellar liposomes stored. (A) room temperature, (B) room temperature and O_2-free atmosphere and (C) 4°C; egg yolk lecithin (*——*), egg yolk lecithin:cholesterol, 5:1 (Δ- - - -Δ), egg yolk lecithin:α-tocopherol, 1:0·05 (□- - -□), egg yolk lecithin:α-tocopherol, 1:0·2 (O— — — — O) and egg yolk lecithin:cholesterol:α-tocopherol, 5:1:0·3 (◇·····◇).

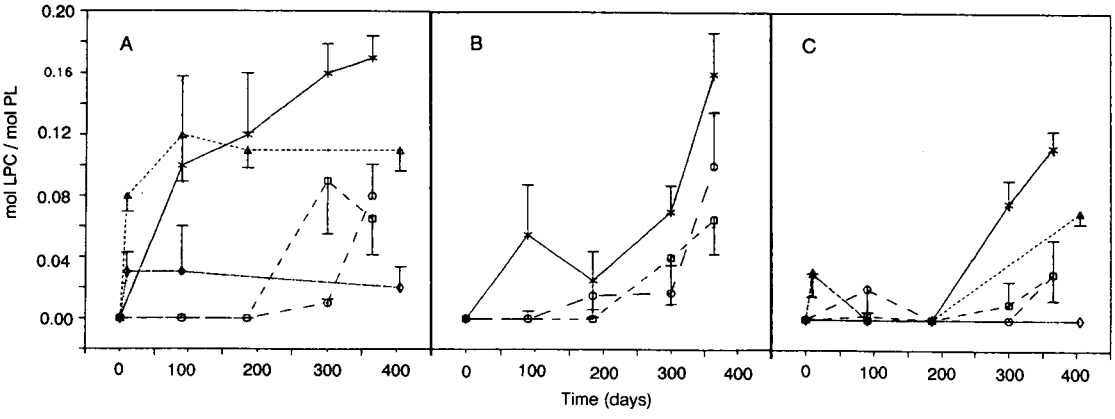

FIG. 2. Lysophosphatidylcholine (LPC) content of stored liposomes (PL). (A) room temperature, (B) room temperature and O_2-free atmosphere and (C) 4°C; egg yolk lecithin (*——*), egg yolk lecithin:cholesterol, 5:1 (Δ- - - -Δ), egg yolk lecithin:α-tocopherol, 1:0·05 (□- - - -□), egg yolk lecithin:α-tocopherol, 1:0·2 (O— — — —O) and egg yolk lecithin:cholesterol:α-tocopherol, 5:1:0·3 (◇·····◇).

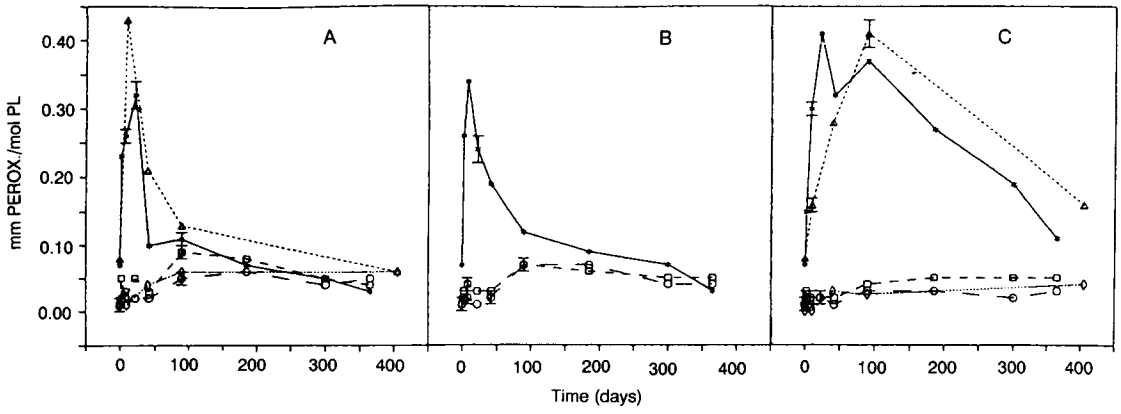

FIG. 3. Lipid oxidation as measured by peroxides in stored liposomes (PL) (A) room temperature, (B) room temperature and O_2-free atmosphere and (C) 4°C; liposomes composed of egg yolk lecithin (*——*), egg yolk lecithin:cholesterol, 5:1 (Δ- - - -Δ), egg yolk lecithin:α-tocopherol, 1:0·5 (□- - - -□), egg yolk lecithin:α-tocopherol, 1:0·2 (O— — — —O) and egg yolk lecithin:cholesterol:α-tocopherol, 5:1:0·3 (◇·····◇).

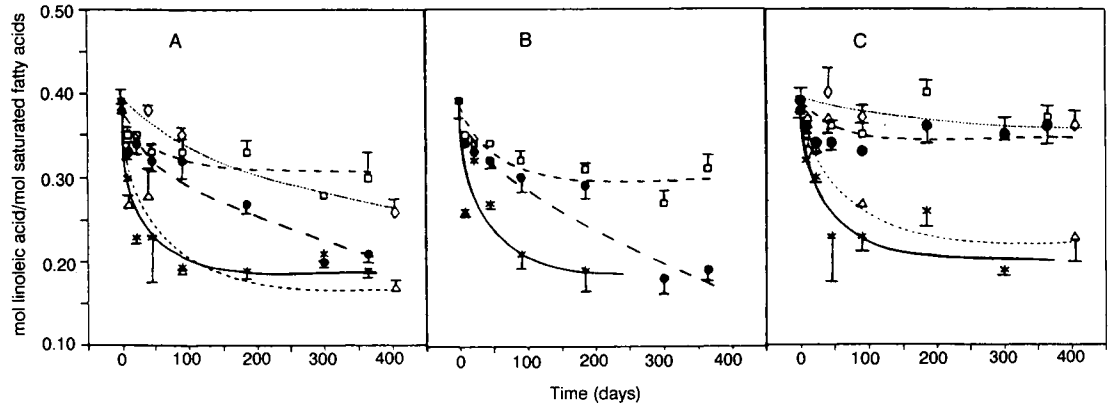

FIG. 4. Linoleic acid compositional changes in liposomes stored at (A) room temperature, (B) room temperature and O_2-free atmosphere and (C) 4°C; egg yolk lecithin (*——*), egg yolk lecithin: cholesterol, 5:1 (Δ- - - -Δ), egg yolk lecithin:α-tocopherol, 1:0·05 (□- - - -□), egg yolk lecithin:α-tocopherol, 1:0·2 (●— — — —●) and egg yolk lecithin:cholesterol:α-tocopherol, 5:1:0·3 (◇·····◇).

content of the liposomes was retained after one year of storage, corresponding to a $t_{\frac{1}{2}}$ greater than 400 days.

We have also measured the evolution of lysophosphatidylycholine in stored liposomes (Fig. 2), since this can affect the physicochemical properties of the phospholipid bilayer.

Another factor affecting liposome stability is oxidation. This we measured with time (Fig. 3) in preparations protected from light and with a low level of heavy metal ions in the presence and absence of O_2.

We also measured fatty acid changes with time in the liposomes. The changes in the content of both palmitic and stearic acids were not significant (data not shown) whereas significant changes were observed for oleic and linoleic acids (Fig. 4).

Discussion

Stability on long term storage is a factor to be taken into account when liposomes are to be used as a pharmaceutical dosage form. One possible way to control stability is to incorporate cholesterol into the lipid structure since it is known to reduce leakage of various solutes through the lipid bilayer when the membrane is in a fluid-like state (Gregoriadis & Davis 1979). Another aspect to consider is liposome oxidation (Frokjaer et al 1984). Oxidation of unsaturated phospholipids and cholesterol may be initiated mainly by the action of light and heavy metals. We have tried to inhibit peroxidation of lipids within membranes by addition of metal chelators,such as EDTA, protection from light, O_2-free atmosphere, the addition of α-tocopherol and low temperature storage (Frokjaer et al 1984; Mowri et al 1984). Crommelin et al (1986) found no effect of α-tocopheryl acetate on liposomes stability; however, α-tocopheryl acetate is much less effective than α-tocopherol in preventing lipid peroxidation (Fukuzawa et al 1981).

Another type of degradation which may occur within membranes is hydrolysis of phospholipid to free fatty acids and lysophospholipids, which can disturb the phospholipid bilayer structure and may disrupt it leading to leakage of encapsulated products.

The mean aim of our work has been to study liposomal compositional changes to improve liposome encapsulating efficiency during long term storage. We have related encapsulating efficiency with those factors responsible for liposome structural disruption.

In this study, egg yolk lecithin was chosen as a model phospholipid, not only because of its widespread use in liposomes, but also because its fluid bilayer is sensitive to alterations in bilayer structure. The introduction of cholesterol in liposomes decreases the rate of leakage during storage because of its effect of rendering the bilayer structure more rigid (Gregoriadis & Davis 1979), but a more dramatic effect on CF retention was seen when α-tocopherol was included in the membranes (Fig. 1, Table 1). CF retention was greater in liposomes stored at 4°C in the presence of O_2 than those at room temperature, although liposomes stored at room temperature but in O_2-free atmosphere were more stable than those stored at room temperature in the presence of O_2.

Lysophosphatidylcholine content in liposomes containing α-tocopherol at room temperature was decreased relative to liposomes of egg yolk lecithin alone and with cholesterol which related with the increased CF retention found for those liposomes (Fig. 2A). The same was true for liposomes stored at room temperature in the absence of O_2 and at 4°C in the presence of O_2 (Fig. 2B, C). Less lysophosphatidylcholine content meant less CF leakage. Although all liposomes containing egg yolk lecithin in the first months of storage do not contain appreciable amounts of lysophosphatidylcholine, there was a marked difference in CF retention amongst these liposomes. Although it is not clear how α-tocopherol stabilizes the phospholipid bilayer, we have suggested that this may happen through specific binding to the phospholipid molecule (Villalaín et al 1986).

Egg yolk lecithin has an appreciable amount of unsaturated phospholipids (approx. 35% oleic acid and 20% linoleic acid) which may undergo oxidation upon storage. Oxidation of phospholipid fatty acids can then destabilize the bilayer structure. Fig. 3 shows lipid oxidation in liposomes. In all liposomes without α-tocopherol there was an abrupt increase in peroxide content during the first few weeks of storage, whereas the peroxide content in liposomes with α-tocopherol was minimal at all times and all conditions.

At low temperature, although there was no inhibition of peroxide formation, peroxide decay, i.e. fatty acid breakdown decreased (Fig. 3C), so that there was an increase in liposome stability (Fig. 1C). This effect could also be seen in the distribution content of fatty acids in the liposomes, where, of those fatty acid types constituting egg yolk lecithin linoleic acid only is reduced significantly in liposomes without α-tocopherol (Fig. 4).

We found that α-tocopherol not only inhibited phospholipid breakdown (lysophosphatidylcholine production), but also inhibited fatty acid peroxidation (Figs 2, 3). The effect produced by α-tocopherol may then be combined with the effect produced by cholesterol on the structure of the bilayer, and the decreased peroxide decay produced by low temperature. The combination of these factors should then make the liposomes much more stable to leakage of encapsulated products (Fig. 1C).

Acknowledgements

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