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Characterization and Stability of Various Liposome-Encapsulated Enoxacin Formulations

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The necessity for antibacterial agents with greater intracellular efficacy has led to the development of endocytosable drug carriers such as liposomes. Enoxacin was selected as a model drug incorporated in various liposome formulations as a therapeutic dosage form using the ethanol injection method and freeze-drying. Liposomal behavior after preparation and stability test was characterized by determining the physicochemical properties of enoxacin encapsulation percent, vesicle size and turbidity. The non-phospholipid formulation of stratum corneum liposomes showed the highest encapsulation efficiency after preparation among nine liposomal formulations. The addition of dissacharides in liposomes also enhanced the encapsulation of enoxacin due to the protection of phospholipid bilayers during the freeze-drying process. The liposomes with negatively charged component and dissacharides showed lower enoxacin leakage after five weeks of storage at 45 °C, suggesting these formulations have high stability in long-term storage. The negative liposomes showed a different behavior than others in their decrease of size and turbidity during storage, possibly due to high surface charges of the negative formulation. Cholesterol stabilized bilayers interacted with plasma and high density lipoprotein (HDL) retained enoxacin in the vesicles. Nevertheless, liposomes with cholesterol caused a hydrolysis problem after incubation with normal saline. The formulation with trehalose not only showed high stability in storage but also in plasma and HDL. This suggested trehalose was useful to incorporate with phospholipids to produce a highly encapsulated and stabilized liposomes of enoxacin. This study also demonstrated that thought is required in utilizing turbidity as a direct index of liposomal vesicle size.

Key words enoxacin; liposome; stability; turbidity; plasma

Liposomes are microscopic vesicles consisting of membrane-like phospholipid bilayers surrounding an aqueous medium. The lipid vesicles are formed spontaneously when phospholipids are hydrated in aqueous medium.[1] Because of their entrapping ability, liposomes are being considered for use as drug-carrying structures or vesicles. The treatment of infections caused by obligate or facultative intracellular microorganisms is difficult because most of the available antibiotics or antibacterial agents have either poor intracellular diffusion and retention.[2] This problem can be conquered by using antibacterial agents with liposomes to develop endocytosable drug carriers. The need for intracellular targeting of antibiotics and antibacterial agents is urgent since intracellular infections are often associated with AIDS.[3] In this study, the newer fluorinated quinolone antibacterial agent of enoxacin is selected as a model drug incorporated with liposome as a drug therapeutic dosage form. Enoxacin exhibits higher antibacterial activity against a broad spectrum of gram-negative and moderate activity against gram-positive bacteria.[4] Clinically, enoxacin is utilized in a wide variety of infections, particularly urinary tract infections and respiratory tract infections.[5]

To use liposome treatment as a pharmaceutically acceptable formulation, one prerequisite is the stability of liposomes during long-term storage and in the blood. The former often limits their clinical use, and the latter determines the carrier potential of the liposome.[6] The encapsulated drug tends to leak out of the bilayer structure and the liposomes aggregate or fuse during storage.[7] On the other hand, a drug will be immediately released into the blood and excreted after administration resulting in the invalidity of liposomes as drug carriers if they are unstable in the plasma.[8] In this study, the storage stability of liposome-encapsulated enoxacin was carried out for the similar condition of the accelerated stability test to a temperature of 45 °C, since liposomes are thermodynamically unstable.[9] The stability of enoxacin liposome formulations in plasma and high-density lipoprotein (HDL) was also tested.

Depending on the selected bilayer composition, liposomes can vary considerably in physicochemical characteristics. Dispersions that have different characteristics facilitated differences in liposome behavior during the stability test. To characterize physicochemical properties of the dispersion such as vesicle size, drug encapsulation percentage which has commonly been determined in the past was assessed in this study. The turbidity of liposome measured by turbidimeter was also evaluated. Turbidity is currently measured by various researchers by the absorbance of an ultraviolet–visible spectrophotometer at a wavelength ranging between 300 and 530 nm.[10–12] However, there is still a part of optical density due to true absorbance, whereas the rest is due to the true turbidity of the liposomes.[13] The turbidimeter is designed for measurement of true turbidity which meets the design criteria of The United States Environmental Protection Agency.

For optimizing and evaluating various liposome-encapsulated enoxacin formulations, nine types of liposome were used in this stability study. In addition to those with various hydrocarbon chain lengths of the fatty acid part of phospholipid, liposomes participated of neutral, positive and negative charges respectively, were also evaluated. Stabilizers and cryoprotectants such as cholesterol and disaccharides were added to liposome formulations due to the enhanced encapsulation.[9,14] Unlike all other biological membranes, the epidermal lamellae

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do not contain phospholipids,[15] so a lipid mixture approximating the composition of stratum corneum (SC) was prepared to assess the physicochemical state and stability of liposomes lacking phospholipids.

Experimental

Materials Enoxacin, dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), distearoyl-L-α-phosphatidylcholine (DSPC), dicetyl phosphate, bovine brain ceramide, cholesterol (CH) 3-sulfate, palmitic acid (PA), CH and HDL were obtained from Sigma Chemical Co. (U.S.A.). Sucrose (SUC) was supplied by Fisons Chemical Co. (U.S.A.). Trehalose (TRE) and stearylamine were provided by Acros Chemical Co. (U.S.A.). All other chemicals and solvents were of analytical grade.

Preparation of Liposomes Liposomes were prepared by a combination of the ethanol injection technique and freeze-drying modified from Lin *et al.*[16] Fifteen mg of enoxacin was added directly to an alcoholic solution composed of 5.1% methanol, 90.3% ethanol and 4.6% isopropyl alcohol of the lipid, and the solution was then rapidly injected into a fivefold greater volume of magnetically stirred pH 5 McIlvaine buffer. In the formulations with cryoprotectant, the buffer was also prepared with 300 mM disaccharide. Then, the liposomal dispersions were frozen in a dry ice–acetone bath and dried in a freeze-dryer (Labconco, U.S.A.) under 10 microHg vacuum at −50 °C for 24 h. The lyophilized liposome powder was then reconstituted and diluted with 60 ml deionized bidistilled water. Two milliliters of the liposome suspension was placed in each sample vial and loaded into the freeze-dryer again. The total lipid concentration was adjusted to 3.9 mM. Various liposomal lipid formulations are listed in Table 1.

Enoxacin Encapsulation Percentage Determination The freeze-dried cake of liposome in the sample vial was reconstituted with 2 ml pH 5 McIlvaine buffer. The aqueous suspension was emulsified in a sonicator bath, resulting in a homogeneous opalescent dispersion. The enoxacin-containing liposomes were separated from untrapped drug by filtering the liposome dispersion under vacuum (5 mmHg) through a 0.025 μm filter (Millipore, U.S.A.);[16] the liposomes were then washed with buffer to complete removal of the free drug.

The amount of entrapped enoxacin was determined by lysis of the liposomes with absolute ethanol. The liposome dispersion was mixed with an equal volume of absolute ethanol to give a clear solution and enoxacin concentration was estimated by HPLC. The percentage of drug encapsulated was calculated by the ratio of enoxacin in liposome vesicles to the total amount of enoxacin in the aqueous suspension.

Vesicle Size Analysis Light scattering measurements were performed with a Coulter submicron particle-size analyzer (Model N4MD, Coulter, U.S.A.). The liposome preparation in the sample vial was diluted with 10 ml bidistilled water to determine the vesicle distribution. The instrumental settings were : temperature, 20 °C; viscosity, 0.01 poise; refractive index, 1.333; reference angle, 90 °C; run time, 200 s; range, 0—3000 nm.

Turbidity Measurement Turbidity determinations were performed with a turbidimeter (Hach, Model 2100AN, U.S.A.). The freeze-dried liposome powder in the sample vial was diluted with 30 ml bidistilled water and transferred to a sample cell, which was placed in the instrumental compartment of the unit and the lid was closed. The turbidity value was subsequently read and recorded. The unit utilized here was nephelometric turbidity units (NTU).

Accelerated Stability Study The stability study was conducted by incubating freeze-dried liposome formulations at 45 °C, 75% relative humidity in an oven. The samples were withdrawn every week for a period of five weeks. Encapsulation efficiency, average vesicle size and turbidity of liposomal suspensions were then determined as cited above.

Association of Plasma Components with Liposomes Liposomes containing 3.9 mM of lipids in 0.4 ml of pH 5 McIlvaine buffer were incubated with 1.6 ml fresh plasma from human blood or HDL (3.30 mg/ml) in normal saline at 37 °C for 60 min under mild shaking in a thermostated bath; the encapsulation efficiency was then detected as cited above. The neat normal saline 1.6 ml incubated with 0.4 ml liposome solution was treated as the control group.

Determination of Enoxacin Content The enoxacin content of various samples was analyzed by a HPLC system consisting of the instruments reported previously.[16] A 12.5 cm long, 4.0 mm inner diameter stainless steel column with LichroCART 250-4 C-18 column (Merck) was used. The drug sample was mixed with a suitable amount of norfloxacin as internal standard. A 0.015 M tetrabutylammonium iodide was added in bidistilled water. The pH was adjusted to 3.0 with 85% (w/v) phosphoric acid. The mobile phase for enoxacin consisting of 90% salt solution and 10% acetonitrile was used at a flow rate of 1.0 ml/min. The column effluent was passed through a UV detector set at a wavelength of 280 nm. The retention time of enoxacin and norfloxacin were found to be 2.8 and 4.0 min, respectively.

Table 1. Lipid Constituents of the Enoxacin Liposomes

No.	Liposome code	Composition (molar ratio)
1	DMPC	DMPC
2	DPPC	DPPC
3	DSPC	DSPC
4	Positive (+)	DMPC : stearyl amine = 9 : 1
5	Negative (−)	DMPC : dicetyl phosphate = 9 : 1
6	SC[a]	Ceramide: CH : PA : CS = 4 : 2.5 : 2.5 : 1[a]
7	DMPC/CH	DMPC : CH = 7 : 3
8	DMPC/SUC	Add SUC for 300 mM in buffer
9	DMPC/TRE	Add TRE for 300 mM in buffer

a) The ratio of SC liposome composition is weight ratio.

Results

Characterization of Various Liposome Formulations of Enoxacin Phospholipids of phosphatidylcholine with saturated fatty acids of C_{14} (DMPC), C_{16} (DPPC) and C_{18} (DSPC) were selected for characterization firstly. The encapsulation, vesicle size and turbidity for these liposomes are compared in Table 2. The encapsulation increased with a decrease in fatty acid chain length. Moreover, the mean vesicle size increased with an increase in alkyl chain length. This trend was consistent with previous papers.[16,17] In the size distribution of DMPC liposomes, it could be seen that the system was composed of two distinct populations, one with a mean size of 100 nm (51%) and the other with a mean size of 301 nm (49%). This phenomenon was not seen for either DPPC or DSPC liposomes. The turbidities of DMPC, DPPC and DSPC liposomes were inversely proportional to the encapsulation efficiencies as seen in Table 2.

Charge incorporation resulted in a significant alteration in average liposome size and a slight alteration in encapsulation. The vesicle size increased in the order of negative < DMPC < positive liposome. The highest encapsulation was obtained for SC liposomes (Table 2) which can be attributed to the presence of CH. As true of the SC liposomes, the encapsulation of DMPC/CH formulation was higher than that of DMPC formulation (Table 2). Addition of CH significantly increased liposomal size.

The encapsulation of enoxacin is enhanced both by SUC and TRE (Table 2). SUC and TRE were effective for the reduction of aggregation or fusion of liposomes from 198 nm to 136 nm and 133 nm respectively. This result was identical to that of Strauss *et al.* who reported that sucrose and trealose were equally efficient at preventing fusion between vesicles of egg phosphatidylcholine and DMPC.[18]

The specific turbidity can act as an indication of the size of particles.[13] In Table 2, the turbidity of various enoxacin liposome formulations shows a similar trend to the vesicle size, but is not exactly the same. This result

Table 2. Characterization of Enoxacin Liposomes by Encapsulation, Vesicle Size and Turbidity after Preparation (Initial) and Five Weeks of Storage

Liposome code	Encapsulation (%)		Vesicle size (nm)		Turbidity (NTU)	
	Initial	After 5 wks	Initial	After 5 wks	Initial	After 5 wks
DMPC	61.55± 6.79	45.72± 8.12	198[a]	500	15.0± 3.2	68.6±17.7
DPPC	54.58± 9.66	29.64± 8.34	657[b]	1130	113 ±26	135 ±22
DSPC	44.11± 5.23	36.99± 6.77	912[b]	1290	131 ±19	158 ±26
Positive (+)	66.83±10.70	57.42± 6.96	451[b]	639	27.2± 4.1	90.1±23.6
Negative (−)	61.20± 9.97	54.11± 6.54	148[a]	51	16.5± 1.7	6.5± 3.6
SC	88.67±15.71	66.40±12.35	364[b]	869	70.5±11.6	176 ±34
DMPC/CH	68.98± 4.22	49.07± 8.87	388[b]	538	82.9±16.0	137 ±16
DMPC/SUC	82.48±16.97	73.33± 6.64	136[a]	241	14.3± 3.3	35 ± 7
DMPC/TRE	71.48±11.14	70.16±15.54	133[a]	230	16.5± 4.1	33.2± 7.9

a) 2 peaks in population. *b*) 1 peak in population.

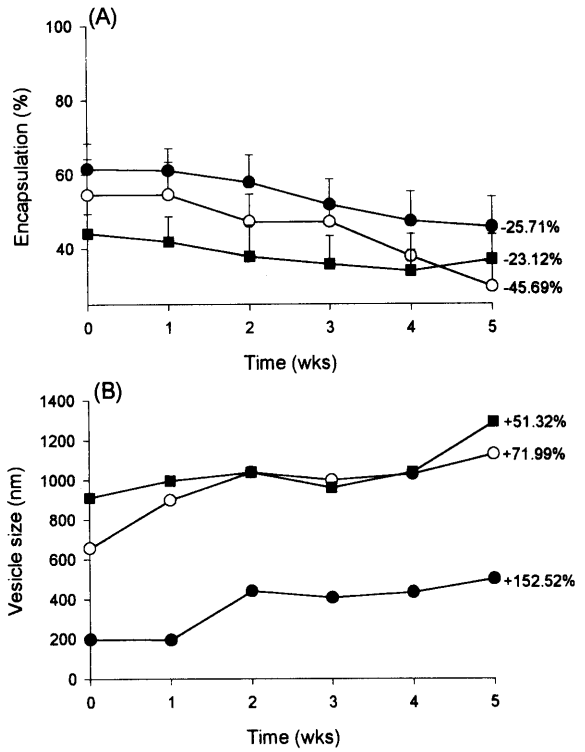

Fig. 1. Accelerated Stability of Enoxacin Liposomes Stored at 45 °C

A: encapsulation ($n=3$); B: vesicle size. Liposomes: ●, DMPC; ○, DPPC; ■, DSPC. The values behind the curves are the percentage changes of encapsulation and vesicle size after five weeks of storage: −, decreased after 5 wks; +, increased after 5 wks.

suggested that care must be taken when considering turbidity of liposomes as an index of vesicle size. In considering of the size distribution of all DMPC-containing liposome formulations, two peaks of the vesicle size population were observed, except in positive and DMPC/CH formulations which had larger liposome size than the other DMPC-containing liposomes and showed a population of only one size distribution.

Accelerated Stability Test A significant loss in enoxacin encapsulation was noted by the influence of fatty acid chain length of phosphatidylcholine in storage as shown in Fig. 1A. Encapsulation loss was associated with an increase in size and turbidity which is a thermodynamically more favorable state as shown in Fig. 1B. As shown in Table 2, the percent of size enlargement after five weeks storage increased following the decrease of fatty acid chain length. Another factor cited here is the change of two size populations in the DMPC size distribution (Table 3). The peak with smaller size remained at a constant level, but the ratio of this population was decreased gradually during storage. The vesicle size and ratio of the higher peak of the population progressively increased.

Table 3. Size Distribution of DMPC Liposomes during Five Weeks of Storage at 45 °C

Time (wk)		0	1	2	3	4	5
Smaller peak	Vesicle size	100	102	117	122	96	102
	Percent	51%	53%	20%	25%	23%	16%
Larger peak	Vesicle size	301	298	521	502	535	575
	Percent	49%	47%	80%	75%	77%	84%

As seen in Fig. 2A, the enoxacin encapsulation efficiency decreased with time after the addition of positive and negative charged components; moreover, the percent of leakage was lower than that of original DMPC formulation after five weeks of storage. It is surprising that the vesicle size and turbidity of negative liposomes decreased progressively during storage as shown in Fig. 2B; this result was quite different from that of the other formulations. The stability of SC liposomes is also shown in Fig. 2. The enoxacin leakage percentage after five weeks showed the same level as DMPC formulation. The same phenomenon was also observed in DMPC/CH formulation although the enoxacin encapsulation was significantly increased after the addition of CH into the liposomes. As shown in Table 2 and Fig. 3, disaccharides not only raise the encapsulation of enoxacin during the process of freeze-drying, but also reduce the possibility of drug leakage within five weeks stability. This phenomenon was particularly obvious in the DMPC/TRE formulation since there was only 1.85% leakage of enoxacin from liposomes after five weeks. The increase of liposomal size and turbidity are also reduced by the addition of disaccarides (Table 2). The curves of DMPC/SUC and DMPC/TRE formulations in vesicle size changes are approximately as depicted in Fig. 3B.

Liposomal Stability in Plasma and HDL Figure 4 shows the release of enoxacin from liposomes after interacting with normal saline and human plasma. There was no enoxacin loss from liposomes for either negative

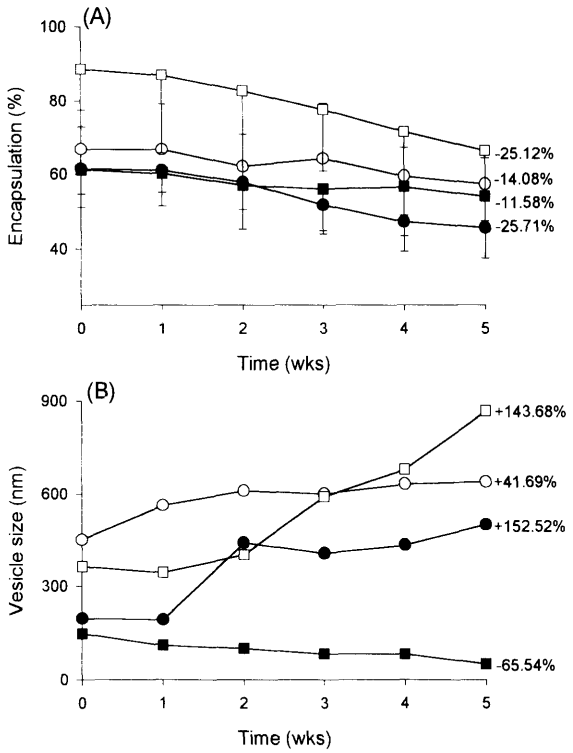

Fig. 2. Accelerated Stability of Enoxacin Liposomes Stored at 45 °C

A: encapsulation ($n=3$); B: vesicle size. Liposomes: ●, DMPC; ○, positive; ■, negative; □, SC. The values behind the curves are the percentage changes of encapsulation and vesicle size after five weeks of storage: −, decreased after 5 wks; +, increased after 5 wks.

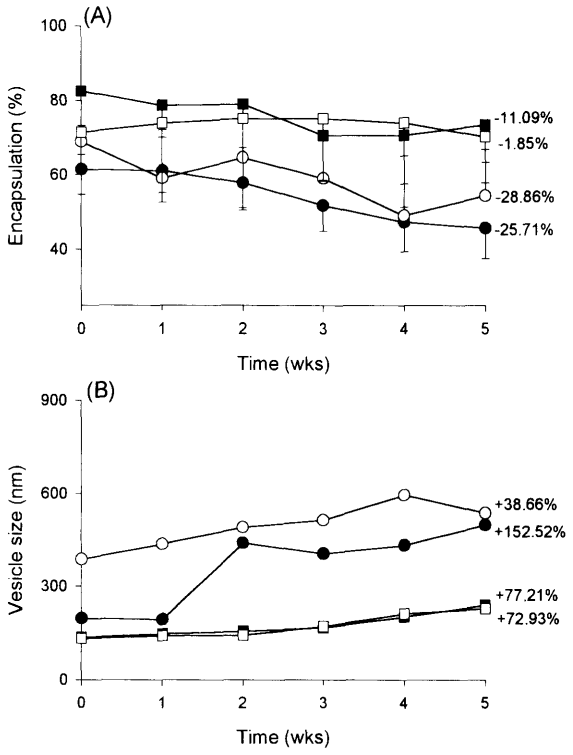

Fig. 3. Accelerated Stability of Enoxacin Liposomes Stored at 45 °C

A: encapsulation ($n=3$); B: vesicle size. Liposomes: ●, DMPC; ○, DMPC/CH; ■, DMPC/SUC; □, DMPC/TRE. The values behind the curves are the percentage changes of encapsulation and vesicle size after five weeks of storage: −, decreased after 5 wks; +, increased after 5 wks.

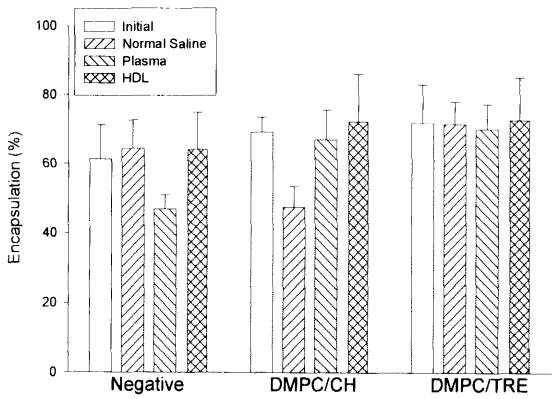

Fig. 4. Encapsulation Efficiency of Enoxacin Liposomes in Normal Saline, Plasma and HDL after 1 h Incubation at 37 °C

Each value represents the mean ± S.D. ($n=3$).

or DMPC/TRE formulations during 1 h incubation in normal saline. However, enoxacin contents was significantly released from DMPC/CH liposomes after incorporation of normal saline as compared with initial encapsulation of DMPC/CH formulations. The leakage of enoxacin from negative liposomes was greatly augmented in the presence of plasma (Fig. 4).

The influence of HDL in the stability was subsequently evaluated. The concentration of human HDL was 3.30 mg/ml in normal saline the same as the HDL concentration in plasma from a healthy volunteer utilized in the plasma stability test. The encapsulations of three formulations tested after incubation with HDL showed no significant difference (t-test, $p>0.05$) compared with the initial encapsulation ratios.

Discussion

Characterization of Various Liposome Formulations of Enoxacin The method of preparing liposomes in this present study was a combination of ethanol injection and freeze-drying. This ethanol injection procedure has no degradatory effect on the phospholipid and shows high encapsulation efficiency for quinolones.[16,19] The limited use of liposome by drug leakage in long-term storage can be overcome by freeze-drying of the liposome suspension. Besides the enoxacin molecules in aqueous medium inside the lipid bilayers, the electrical attractions between bilayers and enoxacin were also important for effective encapsulation in liposomes. DMPC, DPPC and DSPC molecules, in fact, all show the negative surface charge value of −1.[9] Since the amphiphilic enoxacin is positively charged at acid and neutral pH, the pH value of aqueous medium used for determining encapsulation was 5 and the ionic interaction between enoxacin and phosphatidylcholine was formed. This hypothesis was confirmed by Meisner and Mezei: the drug is usually bound to the lipid bilayers in liposomes prepared by ethanol injection–freeze drying method.[20]

Increase in turbidity is associated with shrinkage or aggregation of liposomes. The liposome shrinkage reflects the water permeability of the liposomal membranes.[21] Hence an increase of turbidity may indicate the destabilization of the bilayer membrane structure. This could explain the reason that the turbidities of DMPC, DPPC

and DSPC liposomes were inversely proportional to the encapsulation efficiencies as seen in Table 2. Stearylamine and dicetyl phosphate as the positive and negative membrane component respectively were added to formulations. Since DMPC embraces the negative charge,[9] this addition of positive and negative component resulted in the neutralization and increase in surface potential, respectively.[22] This phenomenon may explain the trend of vesicle size in these three formulations, for the presence of negatively charged phospholipids tended to increase the interbilayer distance owing to electrostatic repulsive forces.[23]

The composition of SC liposomes was chosen as a close approximation to the composition of SC lipids. It is well known that incorporation of CH, a main component in SC, increases the rigidity of fluid state liposomal bilayers.[24] Moreover, experiments with model membranes have shown that CH dramatically reduces the permeability of bilayers to solutes.[25] In view of the vesicle size of SC liposomes, appreciable difference was observed as compared with previous research ranging from 20 to 200 nm.[26,27] The different preparation processes could explain the various vesicle size distribution even the weight ratio of SC liposome composition was all the same.

Addition of CH to liposomes reduced the DMPC content from 100% (DMPC formulation) to 70% (DMPC/CH formulation), inducing more aggregation and leakage because phosphatidylcholine-enriched liposomes have difficulty aggregating due to the repulsive hydration force between bilayers.[28] Content leakage is always involved in the aggregation or fusion of the liposomal membrane.

Various sugars provided satisfactory cryoprotection because of their ability to maintain liposomes intact during freezing. Accordingly, SUC and TRE were incorporated with DMPC to protect the liposomes during the lyophilization cycle since disaccharides are, in general, superior to other sugars in cryoprotection.[14] This enhancing mechanism is established in the prevention of crystallization and solidification of membranes at low temperature during freeze-drying; upon rehydration water molecules quickly replace the sugars and liposomes appear to reseal before significant leakage occurs.[9] Theoretically, vesicles can be sufficiently stabilized so that 100% of the trapped solute is retained after the addition of cryoprotectant.[14] However, there was still approximately 20% and 30% encapsulation loss in this study, because a small amount of sugar inside the phospholipid bilayers during preparation process is required for more effective stabilization.[29] The appropriate selection of buffer ions may also be critical in minimizing freezing damage to liposomes.[30]

Accelerated Stability Test After five weeks of storage, almost one half of the enoxacin had leaked from DPPC liposomes (Table 2). This high encapsulation loss could because a lipid with a transition temperature between the starting and ending temperature of the system provides a means of releasing packaged material as the lipid passes through its phase transition temperature, so that the vesicle experiences a high degree of leakage.[31] Since the transition temperature of DPPC was 41 °C which was between the storage temperature of 45 °C and room temperature as the sample withdrawn from oven,[17] this phenomenon resulted in the destabilization of DPPC formulation and high encapsulation loss of enoxacin.

The transmembrane gradient of the surface pressure increased with decreasing diameter of the liposome.[6] To minimize this pressure,[32] smaller liposomes such as DMPC spontaneously aggregate and fuse with each other as shown in Fig. 1 and Table 2. It is surprising that the vesicle size and turbidity of negative liposomes decreased progressively during storage (Fig. 2B). It is our opinion that negative liposomes may provide high surface charges on the bilayers from both DMPC and dicetyl phosphate. The influence of high surface charges on liposome morphology and stability may cause shape changes and spontaneous formation of unilamellar vesicles from multilamellar ones due to the contributions of Gaussian curvature.[33] This mechanism could explain why the vesicle size and turbidity of negative liposomes were decreased significantly but enoxacin leakage was barely reduced after five weeks of storage.

Lipids from biological sources such as bovine brain ceramide in SC liposomes typically contain significant levels of polyunsaturated fatty acids and therefore are inherently less stable than the other phospholipids.[31] SC liposomes tended to flatten when water leaves the vesicles which was similar to the process of freeze-drying. As SC liposomes flattened to form membraneous disks, they fused edge-to-edge to form tubular as well as large disklike structures gradually.[26,29] This confirmed the result of the present study that SC liposomes greatly increased in size and turbidity when stored in a lyophilized powder form.

The result of DMPC/CH liposomes demonstrated that CH could not provide a stabilization capacity for liposomes during long-term storage since CH is readily oxidized, which creates a stability problem.[31] When liposomes are lyophilized in the presence of trehalose, intact liposomes are obtained following rehydration and retain most of their original contents. This leads to the ability that liposomes may be stored for extended periods in this dehydrated state.[29] The prevention of liposomal aggregation by SUC and TRE was attributed to saccharides and polysaccharides being able to induce steric repulsion among liposomes by coating or adsorbing the liposome surface.[33] This mechanism was quite different from the ability to prevent aggregation by charged components which induced electrostatic repulsion.

After the characterization of vesicle size and turbidity of various liposome formulations during the accelerated stability test, it was found that liposomes with larger mean particle size had a larger turbidity value. The relationship between these two parameters was then considered to determine whether or not the vesicle size of enoxacin liposomes increased linearly with increase of turbidity. After calculation of the correlation coefficient, however, the r value only showed a level of 0.86, it indicating that turbidity cannot be regarded as a direct index of liposomal vesicle size.

Liposomal Stability in Plasma and HDL Stability of liposomes in the blood stream dictates the pharmacokinetics of a drug in a liposome formulation. Both negative

and DMPC/TRE liposomes were used in this plasma stability test since both formulations showed lower leakage of enoxacin after long-term storage at 45 °C. In addition, DMPC/CH liposomes were also used in this study since CH improves the stability of the bilayer membrane in the presence of plasma.[6,9,34] The result of DMPC/CH liposomes indicates that the hydrolysis of DMPC liposomes does not depend on the changes in bilayer rigidity induced by CH.[35]

This is possibly because a net negative charge in liposomes behaves with high affinity to the plasma proteins, resulting in the instability of negative formulation during incubation.[36] Another explanation is that phosphatidylcholine is stable in its bilayer structure since it has two hydrocarbon anchors to form a phospholipid layer.[8] Accordingly, negative liposomes were less stable than DMPC/TRE ones due to the reduction of DMPC content in a negative formulation. The encapsulation of enoxacin from DMPC/TRE liposomes remained unchanged during 1 h incubation in plasma thus preventing direct interbilayer contact by disaccharides.[14]

It is conceivable that HDL attacks only the outermost layer of liposomal vesicle resulting in a higher stability in HDL than in plasma for negative liposomes.[37] Another factor may be that HDL was not the main factor disrupting the structure of liposomes with dicetyl phosphate. Previous work has shown that a number of proteins other than HDL may associate with liposomes: albumin, immunoglobulins and fibronectin are found to attach to the liposome surface composed of phosphatidylcholine and negative charged additives, *e.g.* phosphatidylserine.[38] The high stability of DMPC/CH liposomes in HDL observed in Fig. 4 which is consistent with previous studies suggests no interaction between liposomes with cholesterol or HDL.[34,39] This is presumably the result of the condensing effect of the sterol on the acyl chains of the phospholipids.

In conclusion, the encapsulation of enoxacin liposomes was significantly modified by incorporation with the dissacharides such as sucrose or trehalose. The SC liposomes without phospholipids in a formulation also showed high enoxacin encapsulation. The accelerated stability test was negative and dissacharides liposomes reduced drug loss during storage. Interestingly, the negative formulation showed a different size and turbidity change than the others, possibly because of the high surface charges of negative liposomes. The DMPC/CH liposomes may produce a hydrolysis problem since enoxacin was significantly released after 1 h incubation in normal saline. On the other hand, CH stabilized phospholipid bilayers when incorporating with plasma and HDL, thus retaining enoxacin in liposomal vesicles. The formulation of DMPC/TRE showed high stability in long-term storage, plasma and HDL indicated the addition of TRE not only increased the encapsulation in the freeze-drying process but also stabilized liposome bilayers in various environments. Accordingly, the drug carrier potential of the stabilized liposomes of enoxacin also awaits further exploration. The information obtained in this study will be helpful in development of an enoxacin delivery system by liposomal dosage form.

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