CHAPTER IV

RESULTS AND DISCUSSION

1. Saturation solubility of minoxidil at ambient temperature

The saturation solubility of minoxidil (MN) was experimentally determined in different aqueous media: tri-distilled water, 50 mM acetate buffer pH 4.6, 50 mM borate buffer pH 7.0, 15% propylene glycol (PG) in water (W), and 30 % PG in water. The ambient temperature was monitored and found to be 24-25°C during time of study. The complete solubility data are shown in Table 2.

Table 2. Solubility data of MN in different aqueous media

Time (hour)	Solubility of MN (mg/mL) ± SD (n=3)				
	water	buffer pH 4.6	buffer pH 7	15% PG in W	30% PG in W
1	2.32±0.02	2.96±0.40	2.56±0.12	4.49±0.04	8.51±0.06
3	2.34±0.04	3.68±0.66	2.87±0.08	4.71±0.02	8.76±0.08
6	2.38±0.07	4.10±0.48	2.95±0.03	5.18±0.02	8.95±0.04
9	2.35±0.05	4.50±0.24	3.12±0.01	5.23±0.02	9.22±0.06
12	2.40±0.05	5.11±0.19	3.12±0.03	5.27±0.03	9.56±0.04
24	2.43±0.05	5.17±0.05	3.12±0.02	5.31±0.03	9.60±0.02
48	2.39±0.08	5.21±0.17	3.13±0.02	5.29±0.02	9.60±0.02
54	2.41±0.02	5.21±0.02	3.12±0.02	5.32± 0.05	9.62±0.07
66	2.49±0.03	5.21±0.03	3.12±0.02	5.28±0.01	9.58±0.06
90	2.49±0.07	5.20±0.05	3.12±0.03	5.29±0.03	9.60±0.08
saturation solubility of MN (mg/mL)	2.49	5.20	3.12	5.29	9.60

MN solution at ninety percent saturation in each aqueous medium was used in subsequent studies.

2. Effects of surfactant type and surfactant to cholesterol ratio on vesicle formation and drug entrapment

Form preliminary study, MN niosome formation occurred only when cholesterol (CHO) was present in the formulas. So, CHO was invariably included in all formulations. The total lipid concentration was kept at 100 mg/mL. The lipid was hydrated with 2.2 mg/mL MN in water. This concentration was at approximately 90% of MN saturation solubility in water. The preparation method for this study was the so-called in-process loading method. Tween®20, Tween®80, Span®20, Span®40, Span®60, Span®80, Span®85, and POE-10 were used as surfactants in MN niosomes preperation. CHO was included at a 1:1 weight ratio. These 8 formulations were compared to screen for the most appropriate surfactants that could form the vesicles. From the study, Span®20, Span®40, Span®60, and POE-10 formed vesicles in the presence of CHO (Table 3). CHO at various weight ratios (15, 20, 30, 40, 50, 60, and 70%) was added to these four surfactants. The ratios of surfactant to CHO that allowed complete vesicle formation were 70:30 for Span®40, 60:40 for Span®60, and 50:50 for POE-10, respectively (Table 4). These results are in contrast with many previous reports where niosomes can be formed from a wide range of surfactant to CHO ratio. For example, Span®20 was reported to form vesicles in the presence of 47.5 mg% CHO by film hydration method (Udupa et al., 1993). POE-10 also forms vesicles at various concentrations of CHO including at 85:15 weight ratio (Waranuch et al., 1998). The difference in the energy input into the system might be responsible for the discrepancy seen here. The different methods used undoubtedly yield different input of the energy and thus the difference in ease of lipid dispersion in the aqueous medium.

There is some statistically significant difference between the three formulations in MN entrapment efficiency after an overnight annealing period ($p<0.05$, Table 5). The highest entrapment efficiency was seen with Span®40 formulation.

Table 3. Feasibility of MN niosome formation from various surfactants. Cholesterol was included at 1:1 weight ratio. Total lipid concentration was 100 mg/mL.

Surfactant	Formation of MN niosomes
Span®20	yes
Span®40	yes
Span®60	yes
Span®80	no
Span®85	no
Tween®20	no
Tween®80	no
POE- 10	yes

Table 4. Formation of MN niosomes at various surfactant:CHO ratios. Total lipid concentration was 100 mg/mL. Niosomes were hydrated with 2.2 mg/mL MN in water.

Composition	Formation of MN niosomes						
	surf/CHO ratio by weight						
	85: 15	80: 20	70: 30	60: 40	50: 50	40: 60	30: 70
Span®20:CHO	-	-	+	+	++	+	-
Span®40:CHO	-	-	++++	+++	++	+	-
Span®60:CHO	-	-	+++	++++	+++	+	-
POE-10:CHO	-	+	++	+++	++++	+++	+

++++ = complete vesicle formation

+++ = nearly complete vesicle formation

++ = incomplete vesicle formation with vesicle aggregation and surfactant remnants

+ = incomplete vesicle formation with vesicle aggregation and CHO crystals

- = phase separation and/or no vesicle formation

Table 5. Effect of equilibrating time on entrapment of MN niosomes prepared from various non-ionic surfactants. The ratios of surfactant/CHO were 70:30 for Span®40, 60:40 for Span®60, and 50:50 for POE-10. Total lipid concentration was 100 mg/mL. The data are shown as mean ± SD, n=3.

Equilibrating time (day)	Entrapment efficiency (mg% by weight)		
	Span®40	Span®60	POE-10
1	2.84 ± 0.14	1.90 ± 0.10	0.83 ± 0.07
3	2.63 ± 0.17	1.73 ± 0.07	1.05 ± 0.08
5	2.40 ± 0.11	1.69 ± 0.01	1.02 ± 0.05
7	2.30 ± 0.10	1.68 ± 0.02	0.99 ± 0.14
10	2.20 ± 0.20	1.69 ± 0.01	1.01 ± 0.09
p-value	0.00	0.69	0.10

3. **Effects of equilibrating time on drug entrapment**

Sufficient equilibrating time before separation allows niosome vesicles to properly anneal. In this study, the entrapment efficiency (EE) at various time intervals (1, 3, 5, 7, and 10 days) after preparation was monitored. During the storage time, MN could partition among the aqueous phase, the vesicular membrane, and the aqueous core of the vesicles until the system reached equilibrium. From data shown in Table 5, the equilibrating time had an effect on MN entrapment for MN niosomes prepared from Span® 40 ($p<0.05$). This effect was not seen with MN niosomes prepared from Span®60 and POE-10. For Span®40 entrapment efficiency decreased with time, and equilibrium was reached at around day 5. For Span®60 and POE-10, equilibrium seemed to be reached after only one day of equilibration. The reason for these observations might be the difference in the packing structure of the surfactants. (Philippot and Schuber, 1995).

4. **Effects of formulation factors on MN entrapment in niosomes**

4.1. Effects of total lipid concentration on drug entrapment

Total concentration of bilayer-forming components may affect feasibility of vesicle formation. It is usually easier to hydrate bilayer-forming lipids at low concentration, provided that the concentration is kept above the CMC. At higher concentration, hydration process may be less efficient and may result in lipid remnants. In some occasions, other structures rather than vesicles may be formed (Baillie et al., 1985; Urchegbu and Vyas, 1998; Knepp et al., 1990). In addition, liposomes formed at different lipid concentrations may differ in lamellarity (Du Plessis et al., 1992; Agarwal, Katare, and Vyas, 2001), which in turn may affect entrapment efficiency. This experiment was thus designed to determine whether feasibility of vesicle formation and entrapment efficiency would differ when total lipid concentration was varied in the range of 50-200 mg/mL. The results are shown in Table 6 and Figure 2. Vesicles completely formed in all cases. The entrapment of MN in the vesicles, however, was affected by both the total lipid concentration and the type of surfactant. These findings are statistically significant and also some interaction between the two factors from statistical analysis (Table 7). The effect of total lipid concentration could be attributed to the discrepancy in aqueous compartments within the vesicles. At higher lipid concentrations, vesicles could be multilamellar in structure, whereas at lower lipid concentrations they could be unilamellar or oligolarmellar. This has been seen with liposomes prepared by reverse-phase evaporatioon method (Du Plessis et al., 1992). Since MN is also slightly soluble in water (1:500), the aqueous core will contribute largely to the entrapment efficiency of the vesicles. With multilamellar structure, the aqueous compartment will be much reduced and thus the entrapment efficiency (Lasic, 1998). This effect is more clearly seen with hydrophilic solutes where entrapment efficiency is highest with large unilamellar vesicles (Komatsu, et al., 1986; Weiner, Martin, and Riaz, 1989). The effect of surfactant type was more straightforward. Different surfactants differ in their molecular structures. This can affect both the size of the vesicles and the interaction with MN molecules. Both size and interaction of the solute with the lipid bilayer are known to affect solute entrapment in liposome and niosome vesicles (Florence, 1993; Weiner et al., 1989).

Table 6. Effect of total surfactant/CHO concentration on MN entrapment in niosomes. The data are shown as mean ± SD, n=3.

Composition	Entrapment efficiency (mg% by weight) Total surf/CHO (mg/mL)			p-value
	50	100	200	
Span®40:CHO	3.48 ± 0.08	2.91 ± 0.02	2.10 ± 0.12	0.00
Span®60:CHO	3.29 ± 0.29	2.48 ± 0.02	1.63 ± 0.05	0.00
POE-10:CHO	2.03 ± 0.15	1.42 ± 0.00	1.10 ± 0.02	0.00

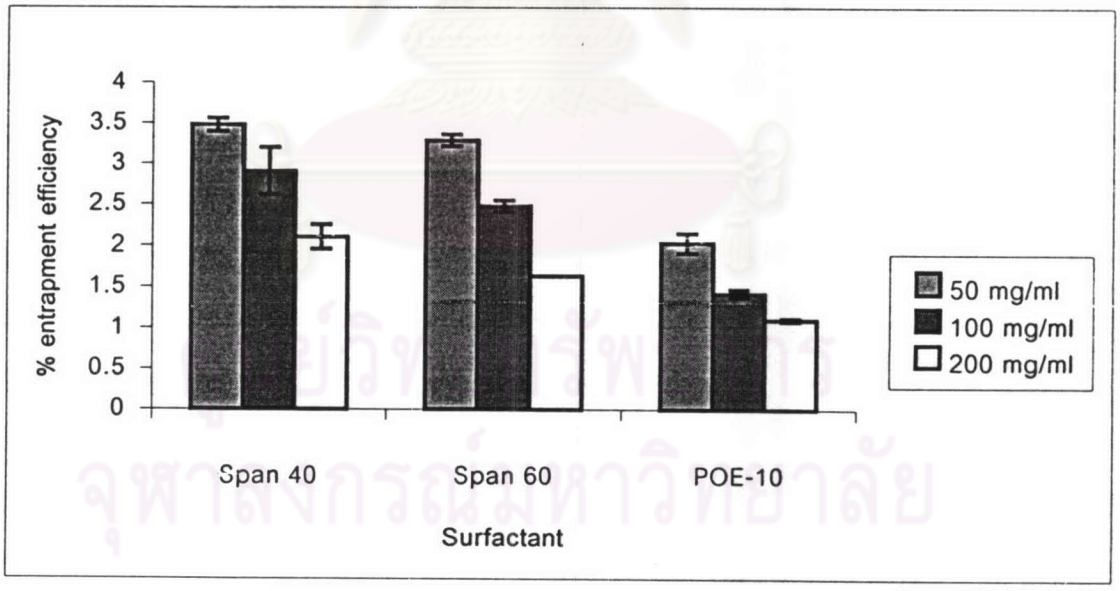

Figure 2. Effect of total surfactant/CHO concentration on drug entrapment

Table 7. Interaction of surfactant type and total surfactant/CHO concentration on entrapment efficiency of MN niosomes prepared by in-process loading method.

Source of variation	Sum of squares	DF	Mean square	F- ratio	p- value
Corrected Model	22.525	8	2.816	450.106	0.00
Intercept	128.271	1	128.271	20505.166	0.00
Surfactant	8.316	2	4.158	664.693	0.00
Conc.	12.384	2	6.192	989.814	0.00
Surfactant* Conc.	1.826	4	0.456	72.958	0.00
Error	0.113	18	0.006253		
Total	150.909	27			

Computed using alpha = 0.05

R squared = 0.995 (Adjusted R Squared = 0.993)

4.2. Effects of stabilizer on vesicle formation and MN entrapment

The stabilizers used in this study were DCP and Solulan®C24. To study the effect of DCP and Solulan®C24 on vesicle formation and drug entrapment, 5% by weight of these compounds was added to the formulations. In the presence of 5% DCP, Span® 40 and Span®60 did not form vesicles even when MN was absent. The mixture became viscous gel instead of milky suspension, implying that other structure rather than vesicles might form. This was not the case with POE-10. Table 8 shows that a much higher MN entrapment efficiency was achieved when 5% DCP was added to POE-10:CHO niosomes. This increase in the entrapment efficiency is statistically significant ($p<0.05$, Table 9). Though MN is an amine drug, ionic interaction with DCP could not explain this observation since MN does not display a net positive charge at the pH studied. An alternative explanation may be the change of vesicular structure when DCP was added to the bilayers. This has been the case with phospholipids where surface charge density of 1-2 $\mu C/cm^2$ results in LUVs instead of MLVs (Weiner et al., 1989). In addition, presence of surface charge also increases the distance between bilayers of MLVs (Namdeo and Jain, 1996). Both scenarios lead to the increased intravesicular aqueous

content. The increased entrapment efficiency can thus be attributed to MN in the intravesicular aqueous phase when DCP was added to the bilayer.

Table 8. Entrapment efficiency of MN niosomes with 5% DCP prepared by in-process loading method. The data are shown as mean ± SD, n=3.

Formulation	Entrapment efficiency (mg% by weight)	
	water	5%DCP
Span® 40:CHO	2.84 ± 0.14	-
Span® 60:CHO	1.90 ± 0.10	-
POE-10:CHO	0.83 ± 0.07	3.83 ± 0.01

Table 9. Multiple comparisons of the entrapment efficiency of POE-10 MN niosome formulations with different stabilizers by one-way ANOVA, with Tukey's HSD test as a post hoc comparison.

Factor (I)	Factor (J)	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Water	DCP	-2.9967*	3.953E-02	0.00	-3.1180	-2.8754
	Solulan C24	-0.6267*	3.953E-02	0.00	-0.7480	-0.5054
DCP	Water	2.9967*	3.953E-02	0.00	2.8754	3.1180
	Solulan C24	2.3700*	3.953E-02	0.00	2.2487	2.4913
Solulan C24	Water	0.6267*	3.953E-02	0.00	0.5054	0.7480
	DCP	-2.3700*	3.953E-02	0.00	-2.4913	-2.2487

*The mean difference is significant at the 0.05 level.

As opposed to DCP, Solulan®C24 did not interfere with vesicle formation of the three non-ionic surfactants. Structurally, this stabilizer is cholesteryl poly(24)oxyethylene ether. The polyoxyethylene side chain is hydrophillic. It stabilizes nisome suspension by steric hindrance, keeping niosome vesicles far apart and preventing aggregation. When 5% Solulan®C24 was added to the formulation, the entrapment efficiency was increased with Span®60 and POE-10 ($p<0.05$, Table 10). The arguments for these observations should be the same as those for the effect of DCP on POE-10 niosomes. Instead of charge repulsion, however, intravesicular steric hindrance might play a key role to increase the internal aqueous volume. The effect of Solulan®C24 was not so clear with Span®40. The results in Table 10 show that, after an overnight annealing, higher entrapment efficiency was seen with Span®40 formulation without Solulan®C24. As observed in Section 3 of this chapter (Table 5), however, entrapment efficiency of Span® 40 niosomes decreased with time, and equilibrium was not reached until day 5. What was seen here could be the result of premature comparison before the equilibrium was established. On the other hand, the mean size of Span®40 (HLB= 6.7) formulation, deducing from the HLB value, was expected to be larger than that of Span®60 (HLB= 4.7) (Namdeo and Jain, 1996). If niosome vesicles have relatively large vesicle size, the effect of its steric hindrance on entrapment efficiency might be obscured, especially for unilamellar or oligolamellar vesicles. The intravesicular aqueous core of large vesicles should be able to accommodate the hydrophilic polyoxyethylene chain of Solulan®C24. In that case, addition of Solulan®C24 should have negligible effect in increasing the intravesicular aqueous space from the aspect of steric hindrance/repulsion. This could be the situation with Span®40 where increase in entrapment efficiency was not evident.

Table 10. Entrapment efficiency of MN niosomes with 5% Solulan®C24 prepared by in-process loading method. The data are shown as mean ± SD, n=3.

Formulation	Entrapment efficiency (mg% by weight)		p-value
	water	1.63 ± 0.05	
Span®40:CHO	2.84 ± 0.14	0.11 ± 0.02	0.05
Span®60:CHO	1.90 ± 0.10	2.66 ± 0.05	0.05
POE-10:CHO	0.83 ± 0.07	1.46 ± 0.04	0.05

4.3. Effects of modification of the aqueous phase on niosome formation and MN entrapment

4.3.1. Effects of propylene glycol

Generally, in MN solution, propylene glycol (PG) is used to increase solubility of the drug. Since the increased MN concentration in the aqueous phase should result in higher drug entrapment in the vesicles, either 15% PG or 30% PG in water was used to prepare MN niosomes. With 30% PG, niosome formation was negated. POE-10 niosomes did not form because POE-10:CHO became completely dissolved in the medium. Span®40:CHO and Span®60:CHO separated out during the preparation process. Thus, it was not feasible to use 30% PG as the aqueous phase for the three non-ionic surfactants studied. When 15% PG was used, niosomes were formed with all three surfactants. The entrapment efficiency was increased in all cases (Table 11). This should be attributed to the higher MN concentration in the aqueous phase. Besides increase in MN concentration in the aqueous phase, difference in extent of vesicle formation and/or vesicular structure might play some roles. The latter might be induced by the change in polarity of the medium with PG. Since POE-10:CHO solubility seemed to be higher in PG solution than in water, the CMC should be accordingly higher. This might result in less of the lipids participating in vesicle formation. Hence, the entrapment efficiency did increase but was in some part offset by the lower number of vesicles present. On the other hand, more lipid moleclues should contribute to vesicles formation with Span®40:CHO and Span®60:CHO, resulting in much more entrapment efficiency values seen with these two surfactants.

Table 11. Entrapment efficiency of MN niosomes prepared in various compositions of the aqueous phase by in-process loading method. The data are shown as mean ± SD, n=3.

Formulation	Entrapment efficiency (mg% by weight)			p-value
	water	15% PG in W	30% PG in W	
Span®40:CHO	2.84 ± 0.14	4.39 ± 0.05	-	0.05
Span®60:CHO	1.90 ± 0.10	4.71 ± 0.06	-	0.05
POE-10:CHO	0.83 ± 0.07	2.09 ± 0.14	-	0.05

4.3.2. Effects of pH of the aqueous phase

In this study, all MN niosome formulations were prepared at 90% saturation concentration of MN in tri-distilled water, 50 mM acetate buffer pH 4.6, and 50 mM borate buffer pH 7.0. Table 12 shows that POE-10 could form vesicles in all three media, whereas Span®40 and Span®60 formulas failed to form vesicles in acetate buffer pH 4.6. In the latter cases, incompatibility was evident. The incompatibility was attributed to the low pH value since a similar result was seen when acetate buffer was substituted by hydrochloric acid solution at the same pH. POE-10 was more resistant to the low pH probably because it contains an ether linkage instead of an ester linkage in Span® surfactants.

For POE-10, the entrapment efficiency increased proportionally with increased MN concentrations in the three media, with acetate buffer giving the highest entrapment. This indicates that most of MN in niosomes was in the aqueous part. At pH 7.0, MN displays no net charge. At pH 4.6, which is its pKa, half of the drug should display a net positive charge. Thus, MN molecules embedded in the bilayer should be reduced since the ionized molecules were less hydrophobic and should partition into the aqueous phase (Weiner et al., 1989; Betageri, Jenkins, and Parsons, 1993; Sharma and Sharma, 1997). The reduced MN in the bilayer was again offset by the much higher concentration of the drug in the aqueous medium. Therefore, the entrapment efficiency increased as the pH decreased. The same trend was seen with Span®60. Span®40, however, gave lower entrapment in 50 mM borate buffer pH 7.0 though MN solubility was

higher in this medium. Changes in vesicle size and/or structure might be responsible for this observation. Large vesicles were absent when Span®40 niosomes were formed in borate buffer. This finding was unexpected and could not be explained by the pH difference since MN solution in water also had a pH of 7.0.

Table 12. Entrapment efficiency of MN niosomes prepared in various buffer by in-process loading method. The data are shown as mean ± SD, n=3.

Formulation	Entrapment efficiency (mg% by weight)			p-value
	water	borate buffer pH 7	acetate buffer pH 4.6	
Span®40:CHO	2.84 ± 0.14	1.42 ± 0.01	-	0.05
Span®60:CHO	1.90 ± 0.10	2.39 ± 0.03	-	0.05
POE-10:CHO	0.83 ± 0.07	1.70 ± 0.03	2.65 ± 0.02	0.03

5. Effects of preparation methods on MN entrapment in niosomes

In this section, the methods of preparation were compared. In the in-process loading method, MN was added to the aqueous phase before vesicles were formed. The drug was expected to reside in the aqueous part of the vesicles as well as to partition into the lipid bilayer owing to its marginal partition coefficient (1.24) (Dennis, 1988). However, it would be beneficial if blank niosomes could be prepared, and the desired drug was loaded later. This would make niosomes much more flexible as a drug carrier system. This latter method is referred to as the passive loading method here. If partitioning was efficient, the resultant entrapment efficiency would be comparable between the two methods. The total concentration of MN was kept at 1.1 mg/mL instead of 2.2 mg/mL due to the dilution factor used in the passive loading method. The entrapment efficiency obtained from the two methods of preparation at various time intervals is displayed in Tables 13-15.

Table 13. Entrapment efficiency of Span® 40 MN niosomes prepared by in-process loading passive loading methods. The data are shown as mean ± SD, n=3.

Time (day)	Entrapment efficiency (mg% by weight)		p-value
	in-process loading	passive loading	
1	2.06 ± 0.08	0.86 ± 0.00	0.05
3	2.01 ± 0.09	0.97 ± 0.03	0.05
5	2.09 ± 0.13	1.23 ± 0.08	0.05
7	2.05 ± 0.12	1.42 ± 0.00	0.04
10	2.16 ± 0.00	1.55 ± 0.01	0.03

Table 14. Entrapment efficiency of Span® 60 MN niosomes prepared by in-process loading and passive loading methods. The data are shown as mean ± SD, n=3.

Time (day)	Entrapment efficiency (mg% by weight)		p-value
	in-process loading	passive loading	
1	1.26 ± 0.09	1.23 ± 0.01	0.51
3	1.28 ± 0.06	0.99 ± 0.04	0.05
5	1.23 ± 0.10	0.91 ± 0.10	0.05
7	1.19 ± 0.15	0.96 ± 0.02	0.05
10	1.18 ± 0.14	0.95 ± 0.04	0.05

Table 15. Entrapment efficiency of POE-10 MN niosomes prepared by in-process loading and passive loading methods. The data are shown as mean ± SD, n=3.

Time (day)	Entrapment efficiency (mg% by weight)		p-value
	in-process loading	passive loading	
1	0.84 ± 0.04	0.80 ± 0.02	0.18
3	0.82 ± 0.01	0.77 ± 0.05	0.18
5	0.83 ± 0.03	0.78 ± 0.00	0.04
7	0.83 ± 0.03	0.78 ± 0.00	0.07
10	0.91 ± 0.01	0.79 ± 0.01	0.04

The results show that partitioning did occur since in the passive loading method MN was found associated with niosome vesicles in appreciable amounts. Partitioning was rather rapid for Span® 60 and POE-10. The entrapment efficiency comparable to that of the in-process loading method was obtained at day 1 ($p>0.05$). On the contrary, partitioning was much slower with Span® 40. For Span® 40, the in-process loading method gave higher entrapment efficiency than that of the passive loading method at all times. Significant difference was seen between the two methods even at day 10 ($p<0.05$). Much less discrepancy in the entrapment efficiency of the two methods was seen with Span® 60 and POE-10 ($p<0.05$). For these two surfactants, the entrapment efficiency was highest at day 1 and slightly decreased thereafter. It is worth noting that the entrapment efficiency from the in-process loading was rather stable for all surfactants including that of Span® 40. The lower MN concentration in the aqueous phase in this experiment might have made the Span® 40 system reach equilibrium faster than what was seen in Section 3 of this chapter. It might facilitate partitioning of MN molecules associated with the outermost bilayer into the aqueous phase, probably by increasing the concentration gradient.

Although the passive loading method appeared to be feasible for drugs with appropriate partition coefficients, one should bear in mind a potential problem with

stability of the system. From this present study, aggregation of vesicles was evident at prolonged period of time (after day 10). This could come partly from the use of formulations that had not been optimized. To avoid aggregation, inclusion of a stabilizer, such as DCP or Solulan® C24, should be considered.