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Effects of Cholesterol and Charging Additives on Stability of Curcumin Niosomes

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Abstract: The objective of this study was to evaluate the effects of addition of membrane additives, cholesterol (chol) with further molecular charges modification using Dicetylphosphate (DCP) and Stearylamine (SA) on stability of curcumin niosomes. Niosomes with entrapped curcumin were prepared by thin-film hydration method. This vesicular drug carrier was constructed with mixture of non-ionic surfactants, Span 60/Tween 60 with addition of various ratios of chol. Further modification to niosomes was conducted by addition of DCP and SA. The niosomes were then characterized in terms of morphology, vesicle size, entrapment efficiency and stability upon storage. The results show that niosomes with ratio of surfactants to chol 1:1 have the highest curcumin entrapment efficiency and the largest vesicle size distribution (136-186 nm) compared to the other samples. On the other hand, the lowest content of chol (ratio 5:1) produced the smallest size distribution of particles ranging between 129-181 nm. Niosomes of ratio 1:1 have the highest entrapped curcumin (97.72%) after 7 days storage, indicating best stability compared to samples of other formulations. The addition of charging additives, DCP and SA did not have any effect in maintaining the stability of curcumin niosomes during short-term storage. The data show that curcumin can be successfully formulated in niosomes and the addition of single additive, chol is sufficient for maintaining the stability of curcumin niosomes constructed from mixture of Span 60 and Tween 60.

Key words: Niosome, curcumin, cholesterol, dicetylphosphate, stearylamine, additive

INTRODUCTION

Curcumin ($C_{21}H_{20}O_6$) is a natural yellow compound which typically found in *Curcuma longa* and has been regarded as a natural polyphenolic antioxidant (Naksuriya *et al*., 2014). It has received attention in cancer treatment study mostly due to its antioxidant, anti-inflammatory, antitumoral, apoptosis-inducing and antiangiogenesis effects. However, curcumin has poor bioavailability due to poor absorption, low stability, rapid metabolism and rapid systemic elimination (Siviero *et al*., 2015). To overcome this limitation, one of the strategies that could be used is by encapsulating curcumin in drug carriers. This technique is able to improve curcumin's bioavailability by providing superior properties such as longer circulation in blood systemic, better cellular permeability and stronger resistance to metabolic processes.

Niosome is one of the promising carriers for curcumin. This carrier has a bilayer vesicles structure and is formed by the hydration of mixture of non-ionic surfactant, cholesterol (chol) and phosphate in an aqueous media (Khan and Irchhaiya, 2016). It has the ability to entrap hydrophobic and hydrophilic drugs in between the bilayers and in its core, respectively, just like liposome. However, niosome owns more advantages compare to liposome such as greater chemical stability, higher purity, content uniformity, low cost could be synthesized using various types of non-ionic surfactants and required no special conditions of handling and storage. Moreover, the niosome base surfactants are biodegradable, biocompatible and non-immunogenic with the human body (Patel *et al*., 2012).

One of the important components in niosome formulations are the membrane additives. These substances are required to stabilize the niosomes. Besides acting as stabilizer, membrane additives also could affect the membrane permeability, encapsulation efficiency, bilayer rigidity and ease of rehydration of freeze dried niosomes. The most common additive found in niosomal

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systems is chol which is known to abolish the gel to liquid phase transition, resulting in less leakiness of the vesicles. Charged molecules compounds such as Dicethylphosphate (DCP) and Stearylamine (SA) which are negative and positive charged respectively, also play roles as the membrane additives in niosomal synthesis. Usually, these chemicals are added in order to prevent aggregation between the niosomes and lead the enhancement of physical stability of this carrier (Seleci *et al*., 2016).

To date, none have reported on the effect of addition of chol with DCP or SA on the stability of curcumin niosomes produced from mixture of non-ionic surfactants, Span 60 and Tween 60. The present study seeks to evaluate the influences of chol addition at three different ratios in curcumin niosomes formulations with further addition of molecular charges represented by DCP and SA on the size distribution, entrapment efficiency and stability properties of the prepared vesicles. The three selected ratios of surfactants to chol being studied were 1:1, 3:1 and 5:1. The stability of curcumin niosomes were investigated at 3 and 7 days of storage based on changes in particles size, reduction of drug entrapment and values of zeta potential.

MATERIALS AND METHODS

Materials: Curcumin, sorbitan monostearate (Span 60), polyoxyethylene sorbitan monostearate (Tween 60), chol, SA and DCP were obtained from Sigma-Aldrich Co., Ltd. Chloroform and methanol was purchased from Merck.

Preparation of curcumin niosomes: Curcumin niosomes were prepared using thin film hydration method (Taymouri and Varshosaz, 2016; Moghassemi and Hadjizadeh, 2014). A solution of curcumin in methanol (0.400 mg/mL) was added to each ratios (1:1, 3:1 and 5:1) of mixture of Span 60/Tween 60 to chol in chloroform/methanol 3:1 v/v to give a final niosomal dispersion concentration of 50 mg/mL. The ratio of Span 60/Tween 60 was kept constant at 2:1. Solvent was then evaporated under reduced pressure at a temperature of 60°C by a rotary evaporator to obtain a thin film deposit. This was followed by the hydration of the lipid-curcumin film with 10 mL of phosphate buffer solution and swirling of hydrated film at 60°C for 45 min in water bath. Finally, the constructed niosomes with encapsulated curcumin were sonicated for 30 min at 60°C in a water bath sonicator. Similar methods were used for preparation of curcumin niosomes with the addition of DCP and SA. Dicetylphosphate and SA, each were added at the ratio of 0.01 towards surfactant during the initial mixture.

Transmission electron microscopy: An electronic-microscope grid was used as a cover with collodion as an electron-transparent support film. A drop of niosomes suspension was placed on the grid for 1 min to attach and excess niosomes were removed using a piece of filter paper followed by the addition of a drop of 2% uranyl acetate solution to the attached niosomes. The remaining solution was then removed after 1 min and the sample was examined using Transmission Electron Microscopy (TEM) after completely dried (Raslan, 2013).

Dynamic light scattering: Size of niosomes was determined using Dynamic Light Scattering (DLS). The curcumin niosomal suspensions were diluted in 3 mL distilled water and size of vesicles was measured at 25±0.1°C. The data were analyzed using the software provided by the manufacturer.

Drug entrapment efficiency: The drug Entrapment Efficiency (EE%) was used to determine the ability of niosomes to entrap curcumin and it can be expressed as:

$$EE\ (\%) = \left[\frac{\text{Amount of enterrapped curcumin}}{\text{Total curcumin}}\right]\times 100$$

Initially, the niosomal dispersion was centrifuged at 11,000 rpm at 4°C for 60 min to remove free curcumin. The pellet was collected and diluted with 5 mL of distilled water and vortexed. This was followed by the addition of 5 mL of 99% methanol. Sample was then vortexed and centrifuged. The supernatant was then quantified by UV-Vis spectrophotometer.

Stability test: The stability of curcumin niosomes at various temperatures was studied by storing the samples in air-tight sealed vials at Room Temperature (RT) with range 20-26, 14 and -4°C for 7 days. The vesicle size, EE % and zeta potential of samples were determined at 3 and 7 days. The zeta potentials were determined using Zetasizer Nano ZS at field strength of 20 V/cm at 25°C and based on the measurement of the electrophoresis mobility of the charged particle. Charged of the particles and their mean zeta potential values with standard deviation were obtained directly from the obtained data.

RESULTS AND DISCUSSION

Effects of addition of cholesterol on vesicle size: Cholesterol acted as a membrane stabilizing agent in the formulation of curcumin niosomes. The interactions between chol and non-ionic surfactant bilayer usually

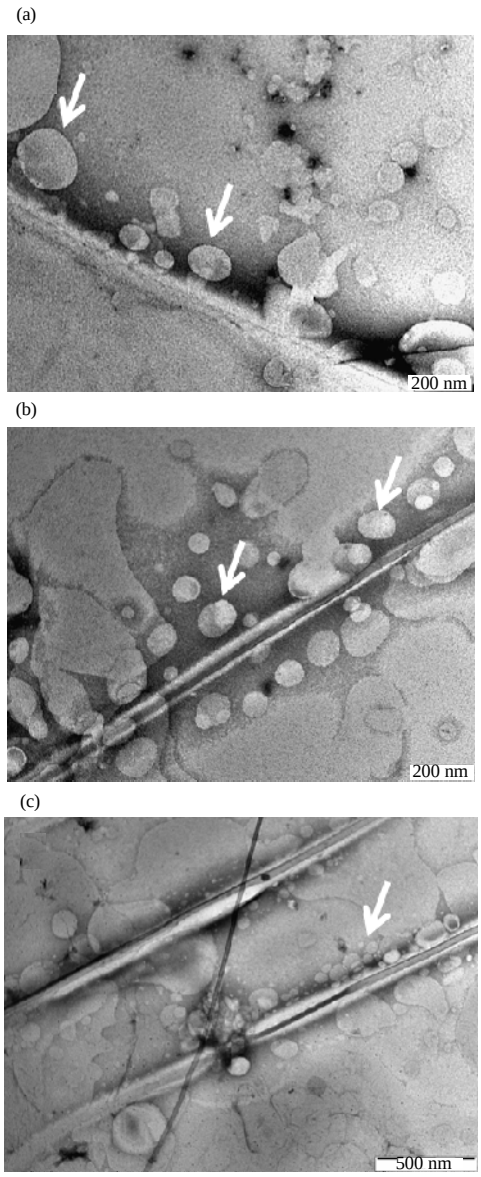

Fig. 1: TEM images of niosomes with surfactants to cholesterol ratio of: a) 1:1; b) 3:1 and c) 5:1. White arrows indicate the curcumin niosomes

depend on the structure of the lipid chain and the hydrophilic head group. The incorporation of chol caused the broadening and eventually disappearance of gel-gel liquid phase transition of lipid bilayer and increased in the degree of orientation order that lead to decrease of the permeability (Bayindir and Yuksel, 2010). Observation by TEM (Fig. 1) revealed well-defined niosomal vesicles with spherical shape in all formulations. It was reported that niosomes containing higher cholesterol content were smaller in size than those containing lower quantity of cholesterol (Chaw and Kim, 2013). However, in the present

Table 1: Size of curcumin niosomes with different ratios of surfactants to cholesterol

Ratios	Minimum size Z-average (nm)	Maximum size Z-average (nm)	Mean size Z-average (nm)
1:1	136.0	186.0	161.0
3:1	132.0	189.5	160.8
5:1	129.0	181.4	155.2

Table 2: Curcumin entrapment efficiency at different ratio of surfactants to cholesterol

Ratios	1:1	3:1	5:1
Initial concentration of curcumin (mg/mL)	0.400	0.400	0.400
Concentration of curcumin entrapped in niosome	0.044	0.024	0.020
EE (%)	11.000	6.000	5.000

study, formulation with ratio of surfactants to chol 5:1 produced smaller vesicles compared to the other two ratios. This observation was confirmed by DLS where Table 1 shows sizes of prepared curcumin niosomes. When the concentration of surfactant decreased, the hydrophobicity properties would also decrease due to the longer alkyl chain and lead to a thicker bilayer structure with high surface tension. Hence, bigger niosomes size would be obtained (Manosroi *et al*., 2003). In the formulation, cholesterol is able to insert itself into the bilayer membrane with its hydrophilic head oriented towards the aqueous surface and aliphatic chain would line up parallel to the hydrocarbon chains in the center of the bilayer. The increased cholesterol content together with reduced non-ionic surfactant content would result in increased hydrophobicity of the bilayer membrane. This might impart disturbance in the vesicular membrane, thus increasing the vesicle radius in a way to establish a more rigid and stable vesicle (Essa, 2010).

Effects of addition of cholesterol on curcumin entrapment efficiency: High encapsulation efficiency of drug would ensure more bioavailability and high concentration of targeted drug which may help in the reduction of dose that related in the systemic side effects (Rangasamy *et al*., 2008). The entrapment efficiency of curcumin at different ratios of chol addition in niosomes formulations is presented in Table 2. The formulation of curcumin niosomes at ratio 1:1 was found to have the highest EE (%). As the cholesterol content in the formulation decreased, the EE (%) became smaller. Cholesterol addition resulted in more rigid bilayer membrane with good barrier function against the water soluble and insoluble compounds, thus decreasing the permeability of the membrane (Junyaprasert *et al*., 2012).

Effects of molecular charged additives on properties of curcumin niosomes: To investigate the stability of niosomes, prepared samples are often stored for at least

Table 3: Size of curcumin niosomes after 3 days of storage

	Average size of niosomes (nm)								
	Chol			DCP			SA		
Ratios	14°C	-4°C	RT	14°C	-4°C	RT	14°C	-4°C	RT
1:1	168.9	152.0	219.0	183.5	161.1	219.2	181.2	163.1	221.2
3:1	171.4	163.1	222.5	191.4	176.8	227.5	190.6	178.4	223.5
5:1	172.8	169.5	231.1	196.1	180.4	241.2	194.2	181.4	221.9

Table 4: Size of curcumin niosomes after 7 days of storage

	Average size of niosomes (nm)								
	Chol			DCP			SA		
Ratios	14°C	-4°C	RT	14°C	-4°C	RT	14°C	-4°C	RT
1:1	179.1	169.6	254.6	194.2	170.5	264.3	195.2	171.5	262.1
3:1	194.8	193.4	262.5	219.9	182.2	275.5	219.8	183.6	276.5
5:1	203.1	198.9	268.1	226.6	191.0	283.8	227.8	194.0	286.6

a month at various conditions. Although, this allow researchers to understand the potential of formulations for long-term storage, a shorter storage period might be sufficient to understand the feasibility of some designed formulations in achieving desired niosomes. Changes to the amount of retained drug at 3 and 7 days of storage has been reported for Span 60: chol niosomes containing combination of tretinoin and benzoyl peroxide (Gupta *et al*., 2015).

The sizes of curcumin niosomes at 3 and 7 days are shown in Table 3 and 4, respectively. All samples showed increased in size as the period of storage increased. Changes were more significant for samples stored at RT compared to samples stored at 14 and -4°C. During the formation of curcumin niosomes, surfactant head groups would interact with the curcumin and developed charged that created mutual repulsion between the bilayer. When the temperature increased with time, the electrostatic repulsive forced among the vesicles and entropic repulsive forces of the head groups of surfactants decreased. Thus, the stability of curcumin niosomes would decreased and lead to the increasing of size. It was also found that samples from the formulation with ratio of surfactants to chol 5:1 has the highest size increased (48.90%) at RT after 3 days of storage compared to samples of ratio 3:1 (38.37%) and 1:1 (36.02%), indicating low sample stability. Furthermore, the addition of charged additives resulted in increased niosomes size. The inclusion of charged molecules into the bilayers would increase the volume of the aqueous compartment due to interaction between charged moiety and the surfactant head groups. This interaction would develop the charged that create mutual repulsion between non-ionic surfactant bilayers and hence increased the particle size (Rangasamy *et al*., 2008). Dicetylphosphate and SA is negative charged and positive charged compound,

Table 5: Percentage of entrapped curcumin after 3 days storage

	Entrapped curcumin (%)								
	Chol			DCP			SA		
Ratio	14°C	-4°C	RT	14°C	-4°C	RT	14°C	-4°C	RT
1:1	97.45	97.95	96.90	94.90	95.53	94.73	94.90	95.05	94.23
3:1	94.60	97.20	93.01	93.80	97.50	91.24	92.10	95.02	90.81
5:1	96.98	98.26	94.89	97.21	97.33	94.13	94.65	96.94	93.31

Table 6: Percentage of entrapped curcumin after 7 days storage

	Entrapped curcumin (%)								
	Chol			DCP			SA		
Ratio	14°C	-4°C	RT	14°C	-4°C	RT	14°C	-4°C	RT
1:1	97.20	97.72	95.98	94.53	94.98	93.11	93.65	94.01	92.10
3:1	92.90	96.40	90.10	92.11	96.35	84.31	90.10	93.69	83.10
5:1	93.10	94.15	88.66	92.87	93.11	89.58	90.21	92.51	82.89

Table 7: Zeta potential for samples with addition of DCP

	Zeta potential (mV)						
	0 days		3 days		7 days		
Ratio	14°C	RT	-4°C	RT	14°C	-4°C	RT
1:1	-60.3	-50.7	-52.3	-47.1	-38.00	-43.1	-36.8
3:1	-58.3	-32.0	-40.4	-24.0	-21.30	-35.2	-17.5
5:1	-48.8	-20.1	-31.2	-15.6	-8.53	-20.4	-7.8

respectively. Hence, there was slightly different effect in stability of the niosomes containing SA compared to those containing DCP. The addition of SA was found to significantly increase the size of curcumin niosomes. This might be due to the positive charge of SA that was incorporated into the niosome membrane which resulted in the water efflux in the bilayer and causing the separation between the layers. This increased the size and membrane thickness of the vesicles (Bayindir and Yuksel, 2010).

Table 5 and 6 show the percentage of entrapped curcumin in niosomes for samples stored for 3 and 7 days. The highest reduction of percentage of entrapped curcumin was observed in samples stored at RT. On the other hand, there was a negligible amount of reduction of entrapped curcumin in niosomes especially for samples of 1:1 ratio upon storage at 14°C and approximately no reduction at all at -4°C. At low temperature, the fluidity of the bilayer vesicles remained unchanged. Hence, prevented the curcumin from being released and no leakage of drug occurred. This proved that the suitable storage of curcumin niosomes should be at low temperature (-4°C). The addition of DCP and SA gave no significant effect in stability properties in preventing reduction of entrapped curcumin.

Table 7 and 8 shows the zeta potential data on curcumin niosomes with addition of DCP and SA, respectively. Curcumin niosomes with addition of DCP

Table 8: Zeta potential for samples with addition of SA

	Zeta potential (mV)						
	0 days		3 days		7 days		
Ratio	14°C	RT	-4°C	RT	14°C	-4°C	RT
1:1	-53.5	-31.2	-40.9	-24.1	-23.6	-34.8	-18.30
3:1	-37.5	-22.7	-30.1	-20.6	-16.0	-21.2	-9.60
5:1	-32.6	-20.1	-23.2	-17.8	-11.8	-17.8	-4.36

contained sufficiently high charged for electrostatic tabilization by its negative charge values compared to the vesicles formulation with addition of stearylamine. It was known that as zeta potential increased in the negativity charged (more negative than 30 mV), the vesicles particles might repel one another and they became more stable against aggregation (Bayindir and Yuksel, 2010). Hence, it showed that the niosomes can be suspended well in water. The formulation of curcumin niosomes with SA and low concentration of chol showed much lower value of zeta potential. The zeta potential depends on movement of particles (electrophoretic velocities), thus the low zeta potential could be due to possible aggregation of particles (Junyaprasert *et al*., 2012).

CONCLUSION

In this study, the formulation of curcumin niosomes with 1:1 ratio of surfactants to chol was found to have the best EE (%). The addition of charged additives did not have significant effect on maintaining the entrapped curcumin during short-term storage. Hence, the addition of single additive, chol is sufficient for maintain the stability of curcumin niosomes constructed from mixture of Span 60 and Tween 60.

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