

## Effect of Unsaturation on the Stability of C<sub>18</sub> Polyunsaturated Fatty Acids Vesicles Suspension in Aqueous Solution

Yin Yin Teo,\* Misni Misran, Kah Hin Low, and Sharifuddin Md. Zain

Department of Chemistry, Faculty of Science, University of Malaya, 50603, Kuala Lumpur, Malaysia

\*E-mail: yinyinteo@um.edu.my

Received June 18, 2010, Accepted October 21, 2010

Degree of unsaturation in fatty acid molecules plays an important role in the formation of vesicles. Vesicle formation from C<sub>18</sub> fatty acids with different amount of double bonds such as oleic acid, linoleic acid and linolenic acid with the incorporation of 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DPPE-PEG2000) have been examined by TEM. Critical vesicular concentrations (CVC) of the vesicle suspension are determined by turbidity and surface tension methods. The CVC of fatty acids increases when the amount of unsaturation in the alkyl chain increases. On the other hand, stability of vesicle suspension has been examined by using particle size and zeta potential at 30 °C. There was a dramatic decrease in particle size measurement from mono-unsaturation to tri-unsaturation which could be due to the effect of fluidity in the membrane bilayer caused by different degree of unsaturation. The values of zeta potential for vesicles that were formed without the incorporation of DPPE-PEG2000 were in the range of -70 mV to -100 mV. It has been observed that the incorporation of DPPE-PEG2000 to the vesicle reduces the magnitude of zeta potential. However, this phenomenon does not obviously seen in fatty acid vesicles formed by linoleate-linoleic acid and linolenate-linolenic acid. We therefore conclude that the addition of DPPE-PEG2000 does not effectively improve the stability of the linoleate-linoleic acid and linolenate-linolenic acid vesicle at pH 9.0 after the evaluation of their particle size and zeta potential over a period of 30 days. Although the vesicles formed were not stable for more than 10 days, they have displayed the potential in encapsulating the active ingredients such as vitamin E and calcein. The results show that the loading efficiencies of vitamin E are of encouraging value.

**Key Words:** Stability, Unsaturated fatty acid, Vesicles, Zeta potential

### Introduction

Vesicle is an artificial microscopic lipid bilayer membrane separating inner aqueous compartment from outer aqueous environment. It is capable in encapsulating drugs and active ingredients. This capability of vesicle provides a promising technology for protection of loads during storage and delivery to the target site. Dialkyl phospholipids are commonly used in the preparation of vesicles. However, single chain fatty acids have also been reported to be able to form vesicles.<sup>1,2</sup> As phospholipids are relatively more expensive compared to amphiphilic fatty acids, therefore, amphiphilic fatty acids maybe a better replacement for phospholipids.

There are several techniques for the preparation of vesicle. The most common technique to prepare vesicle is dry lipid film hydration. It involves evaporation of solvent from amphiphilic solution which produces a thin surfactant films on the wall of the container. These thin films will be hydrated when they come into contact with warm aqueous environment that later lead to the formation of vesicles.<sup>3</sup> There are reports which suggested that vesicles can be spontaneously produced from surfactant solution without the application of any external stimuli. One of the methods that induce the formation of fatty acid vesicles spontaneously is by changing the pH of fatty acid solution through acid-base titration or by pH-jump technique.<sup>4,5</sup> The kinetics of spontaneous formation and breakdown of the vesicle when subjected to a pH-jump perturbation were rapid over a time scale of a few seconds.<sup>1</sup> However, fatty acid vesicles

are kinetically stable but thermodynamically unstable. Hence, the vesicles formed are polydispersed and their sizes may be varied from small to giant oligolamellars. As reported elsewhere, vesicles of laurate-lauric acid as a saturated short chain fatty acid were found in emulsion<sup>2</sup>. On the other hand, formation of unsaturated long chain fatty acid vesicle from *cis*-9-octadecenoic acid, *cis,cis*-9,12-octadecadienoic acid and docosahexaenoic acid in dilute aqueous solution have also been reported recently.<sup>6-8</sup>

The inherent problem of vesicle suspension is its instability during prolonged storage and thermodynamically sensitive to the surrounding. This will hinder the appropriateness and effectiveness of vesicles in their applications such as in drug delivery systems. Therefore, the incorporation of bulky hydrophilic molecule such as polyethylene glycol, polysaccharides and protein to the vesicle has been studied extensively to increase the stability of vesicle.<sup>9-11</sup> In general, stability can be considered as the ability of vesicles to maintain their particle size and remain suspended in solution with no agglomeration or flocculation during storage time. Thus, by monitoring the particle size and zeta potential of vesicle suspension over a period of time could determine the stability of vesicle. Other factors such as molecular structure of the amphiphilic molecule, molecular rigidity, head group type, hydrocarbon chain length and degree of unsaturation may well contribute to the variation in the vesicle suspension stability during the storage time. Only little works have been reported on the stability of unsaturated fatty acids vesicle. For this paper, we have studied, firstly, the effect of

unsaturation on the stability of the vesicle in aqueous solution namely oleic acid (*cis*-9-octadecenoic acid), linoleic acid (*cis*, *cis*-9,12-octadecadienoic acid) and linolenic acid (*cis*, *cis*, *cis*-9,12,15-octadecatrienoic acid). Secondly, we have also studied the effects after the incorporation of 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-[methoxy(polyethylene-glycol)-2000](DPPE-PEG2000) on the stability of vesicle. Lastly, the loading efficiency studies of calcein as a hydrophilic substance and DL- $\alpha$ -tocopherol acetate as a hydrophobic substance have been evaluated.

### Methods and Materials

**Materials.** Oleic acid (*cis*-9-octadecenoic acid,  $\geq 99.0\%$ ), linoleic acid (*cis*, *cis*-9,12-octadecadienoic acid,  $\geq 99.0\%$ ) and boric acid minimum 99.5% were purchased from Fluka (Buchs, Switzerland). Alpha-linolenic acid (*cis*, *cis*, *cis*-9,12,15-octadecatrienoic acid) and DL- $\alpha$ -tocopherol acetate were from Sigma (St. Louis, USA) with purity  $\geq 99.0\%$  and 96%, respectively. 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DPPE-PEG2000) was from Avanti Polar Lipids Inc. (Alabama, USA). Hydrochloric acid, sodium hydroxide 98% and chloroform of analytical grade were purchased from HMBG Chemicals. Calcein and solvent for HPLC which are methanol, ethanol and acetonitrile of HPLC grade were from Merck. The above mentioned chemicals were used as received. Deionized water with  $18.2 \mu\text{S cm}^{-1}$  was obtained from Barnstead NANO pure<sup>®</sup> Diamond<sup>™</sup> ultrapure water system. Deionized water was further distilled and deaerated under nitrogen gas prior to use.

**Preparation of Stock Solution.** A stock solution of 12.62 mM oleic acid and 27.81 mM NaOH was prepared by mixing 0.90 g of oleic acid into NaOH (0.5000 mL 1.3905 M) solution. Thereafter, the mixture was stirred for 2 hours. The procedure for preparation of stock solution for linoleic acid and alpha-linolenic acid are essentially the same, except that the concentration for each of them has to be adjusted accordingly. On the other hand, preparation of vesicle solution containing DPPE-PEG2000 had been prepared by firstly mixing fatty acid with DPPE-PEG2000 in the mole ratio of 50 to 1 in small amount of chloroform. Secondly, the mixture solution was sonicated in order to dissolve DPPE-PEG2000. It was followed by the removal of the chloroform under reduced pressure by using rotary evaporator. Gel liked mixture was obtained and rehydrated with warm deionised water (50 °C) and NaOH solution to form the colourless solution.

**Titration of the Stock Solution with HCl (1 M).** A series of samples with fixed amount of fatty acid at various pH were prepared by mixing 1.50 mL of stock solution with the appropriate amount of 0.7725 M HCl and deionised water. The mixture was left to vortex for a minute by using Uzusio VTX 3000L vortex mixer before the pH measurement was carried out by a Mettler Toledo pH meter which had been pre-calibrated at the titration temperature with buffer pH 4.01, 7.00 and 9.21. An average of 3 measurements was recorded.

**Transmission Electron Microscopy.** The vesicle images were obtained by using Hitachi H-7100 transmission electron microscope through negative-staining method. The samples were pre-

pared by immersing the formvar-coated copper grid into a drop of the vesicle solution. Thereafter, it was allowed to stand for 10 minutes. The excess of vesicle solution was blotted with filter paper before staining process by using 3% (w/v) phosphotungstic acid. The grid was allowed to stand for another 10 minutes and air-dried. The specimens were viewed and photographed with a transmission electron microscope operating at accelerating voltage of 100 kV.

**Critical Vesicular Concentration (CVC) Determinations.** A series of solutions with different concentration of fatty acid at pH 9.0 in 50 mM borate buffer were prepared. Borate buffer solution was prepared in the manner as mentioned elsewhere.<sup>7</sup> The solutions were filtered through a 25 mm diameter 0.2  $\mu\text{m}$  pore size Minisart<sup>®</sup> NY nylon filter (Germany) prior to measurements. The CVC determinations were carried out at 30.0 °C by *via* tensiometer balance from KRUSS with K12 tensiometer processor *via* Du nuoy ring method. The CVC value obtained was double confirmed by turbidity measurement at the chosen wavelength of 350 nm by employing Varian Cary 50 UV-vis spectrophotometer at same temperature. The cell housing was thermostated by the VARIAN Cary single cell peltier unit with a water circulator water bath.

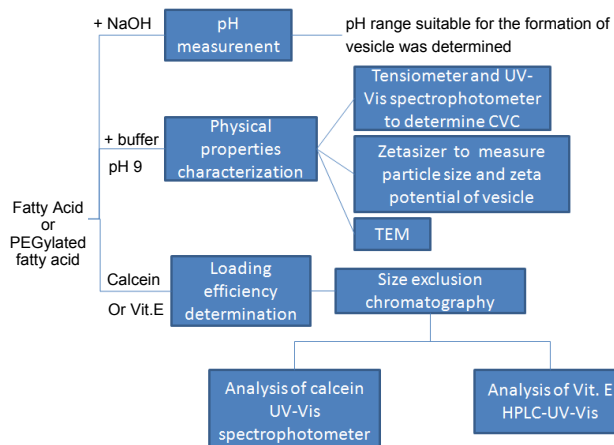
**Particle Size and Zeta Potential Measurement.** The hydrodynamic diameter of the vesicle was measured by the dynamic light scattering (DLS) method. The mean size of the vesicle and zeta potential were estimated by Malvern Nano ZS particle size analyzer from Malvern Instruments Ltd. UK at 30 °C. The solutions were first extruded through 100 nm pore diameters polycarbonate Whatman membranes filter using Lipex Biomembrane extruder prior to the size measurement.

### Studies of Loading Efficiency.

**Encapsulation of DL- $\alpha$ -Tocopherol Acetate:** Fatty acid and DL- $\alpha$ -tocopherol acetate in the mole ratio of 25 to 4 were mixed in  $\text{CHCl}_3$  and subsequently dried under rotor evaporator to remove the  $\text{CHCl}_3$ . This mixture was then blown with stream of  $\text{N}_2$  gas to ensure total removal the trace amount of  $\text{CHCl}_3$  followed by rehydrated with 50 mM borate buffer pH 9.0. The pH of the solution was adjusted to 9.0 by 0.5 M sodium hydroxide and 0.5 M hydrochloric acid.

**Encapsulation of Calcein:** Calcein (0.5 mM) was dissolved in 50 mM borate buffer pH 9.0 then added into a  $\text{CHCl}_3$  solution with 25 mM fatty acid. The mixture solution was kept stirring until all of the  $\text{CHCl}_3$  was eventually evaporated. The resulting mixture was then adjusted to pH 9.0 by NaOH and HCl solution.

**Determination of Loading Efficiency:** Separation of loaded/unloaded species for calcein and DL- $\alpha$ -tocopherol acetate were achieved by gel permeation chromatography technique. The solid phase consists of Sepharose 4B that pretreated with fatty acid solution just above the CVC (mobile phase); which was packed in a glass column of 30 cm  $\times$  1 cm. For sample introduction, 200  $\mu\text{L}$  of the mixture solution was applied. Subsequently every 2 mL of eluent was collected and each fraction was diluted with ethanol to a total volume of 5 mL. Ethanol was applied instead of Triton-X to overcome the self-quenching effect especially for calcein as proposed by Ishii and Nagasaka (2001).<sup>12</sup> In fact, addition of ethanol addresses disruption of the vesicles and fully release those encapsulated species as a consequence.<sup>13</sup> For the determination of calcein concentrations,



**Figure 1.** Schematic figure for vesicle preparation and characterization.

spectrophotometric measurement at 496 nm was employed as suggested by Namani *et al.*<sup>8</sup> On the other hand, the amount of DL- $\alpha$ -tocopherol acetate was analysed by Shimadzu LC-20AT HPLC coupled with an UV detector. A sample of 20  $\mu$ L was injected into C18 reverse phase Purosher<sup>®</sup> Star column (dimension 4.6 mm  $\times$  250 mm and the particle diameter of 5  $\mu$ m) and separated under isocratic flow of mobile phase (10% acetonitrile, 45% ethanol and 45% methanol). The flow rate is 1.0 mL min<sup>-1</sup> and the eluent was monitored at 287 nm. The loading efficiency (%) was calculated as stated in equation 1.

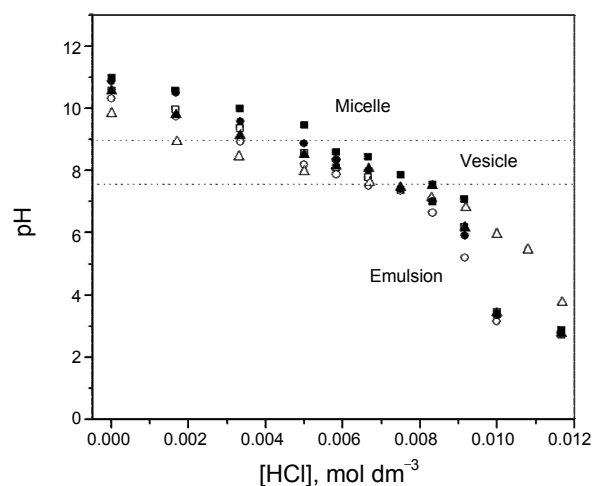
$$\text{Loading efficiency (\%)} = \frac{\text{Absorbance or area of encapsulated material}}{\text{Absorbance or area of amount material}} \times 100 \quad (1)$$

A schematic flow of the current study is illustrated as in Figure 1.

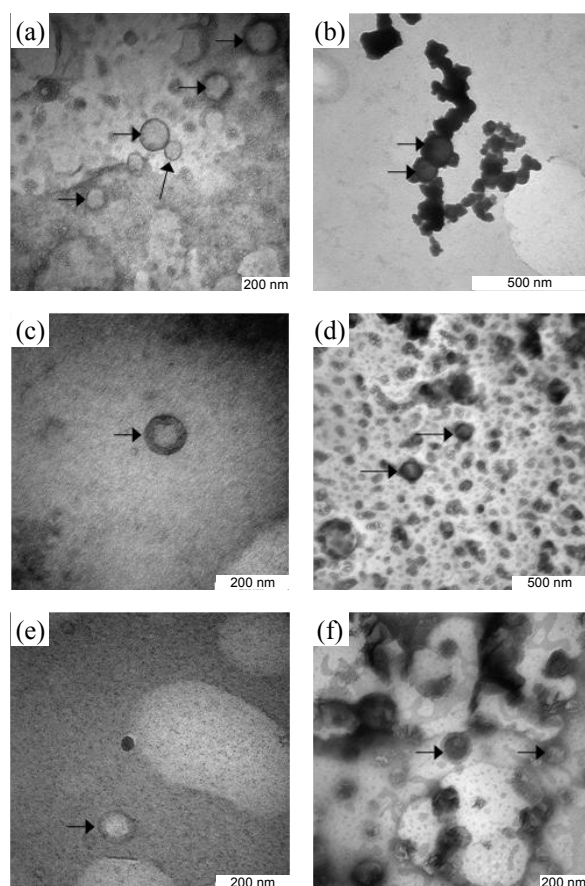
## Results and Discussion

**Titration Curve.** The equilibrium curve of fatty acids as a function of HCl concentration (Figure 2) shows the transparent micellar solutions of deprotonated fatty acid at pH greater than pH 9.5. The presence of vesicles for oleate-oleic acid and linoleate-linoleic acid were observed at pH of the solution between pH 8.0 - pH 9.5 while for linolenate-linolenic acid solution was between pH 7.5 - 9.0. The results obtained were in agreement with those reported elsewhere for vesicle formation by oleic acid and linoleic acid.<sup>7</sup> Nevertheless all of these three types of fatty acids are having almost the same buffering capacity. However, formation of vesicles from linolenic acid has not been studied. On the other hand, incorporation of DPPE-PEG2000 to the vesicle seems did not show significant effect on the trend of equilibrium curve.

The pH region at which vesicles was observed is approximately equal to the pK<sub>a</sub> of fatty acid. According to Kanicky and Dinesh, pK<sub>a</sub> for oleic acid is 9.85, for linoleic acid and linolenic acid are 9.24 for the former and 8.25 for the latter.<sup>14</sup> At these pH,

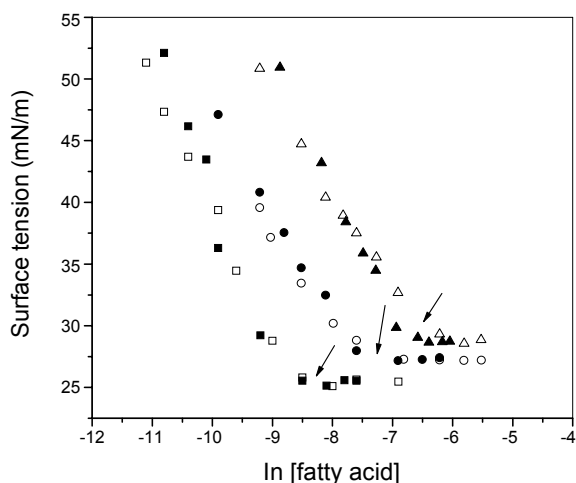


**Figure 2.** Equilibrium curve of fatty acid as a function of added HCl at room temperature (28 °C), (■) 12.5 mM oleic acid, (●) 12.5 mM linoleic acid and (▲) 12.5 mM linolenic acid. Fatty acid with DPPE-PEG2000 were represented by opened symbol.



**Figure 3.** Transmission electron micrograph of (a and d) oleate-oleic acid, (b and e) linoleate-linoleic acid and (c and f) linolenate-linolenic acid at pH 9.0. Figure (a-c) without incorporation of DPPE-PEG2000 while (d-f) with incorporation of DPPE-PEG2000. The presences of vesicles are indicated by arrow.

about half amount of the corresponding acid are ionized. The ionic pair interaction from the ionized and non-ionized single



**Figure 4.** Surface tension as a function of fatty acid concentration, (■) oleate-oleic acid, (●) linoleate-linoleic acid and (▲) linolenate-linolenic acid at 30 °C. Opened symbol indicates the respective fatty acid with DPPE-PEG2000. The arrows in the curve show the CVC of the fatty acids.

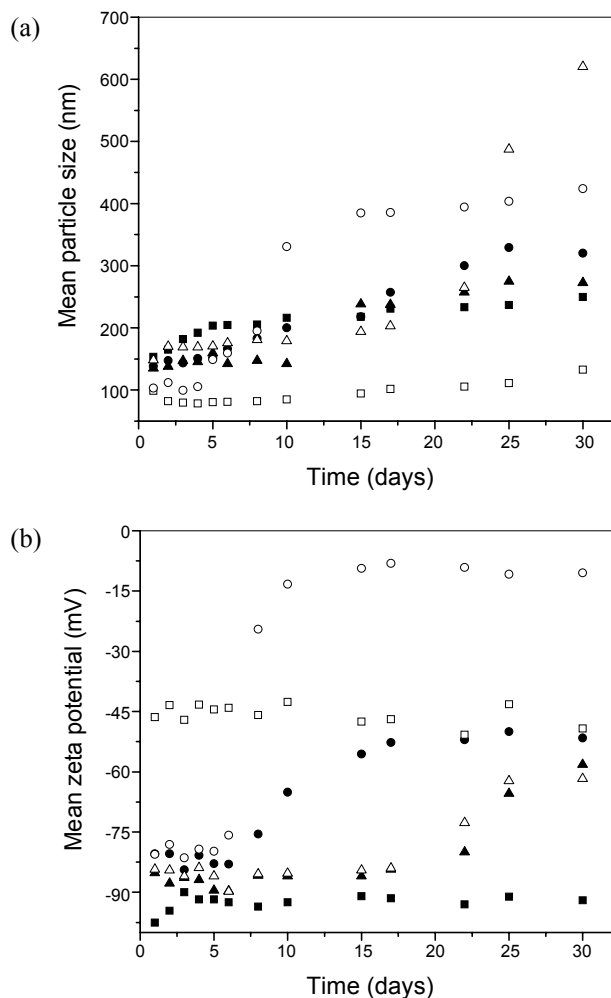
**Table 1.** Summary of CVCs measured by different methods at 30 °C

Fatty acid	CVC, mM	
	By surface tension method	By turbidity method
Oleate-oleic acid	0.13	~ 0.2
DPPE-PEG2000 -oleate-oleic acid	0.17	~ 0.4
Linoleate-linoleic acid	0.51	~ 0.5
DPPE-PEG2000-linoleate-linoleic acid	0.84	~ 1.0
Linolenate-linolenic acid	1.23	~ 1.2
DPPE-PEG2000-linolenate-linolenic acid	1.48	~ 1.6

chain fatty acid molecules brings about an increase in the packing parameters that induced the vesicle formation.

**Transmission Electron Micrograph.** The presence of vesicles in a solution has been confirmed by using TEM. As revealed in Figure 3, negatively stained electron micrographs of the samples prepared from fatty acids mentioned above confirms the formation of vesicles with average diameter of 100 - 250 nm. The vesicle size determined from TEM images are consistent with the size distributions acquired from DLS measurement. Notwithstanding the numerical differences between both measurements, the deviations were not significant.

**CVC Determinations.** CVCs for C<sub>18</sub> polyunsaturated fatty acids include oleic acid, linoleic acid and linolenic acid were determined at pH 9 for comparison purposes. All CVC values were determined at the inflection point of a plot of surface tension as a function of fatty acid concentration as shown in Figure 4. CVC for oleic acid is the lowest whereas linolenic acid has the highest value of CVC as mentioned in Table 1. In general, hydrophobicity of the aliphatic chain will be weaker if the number of unsaturation is higher. Therefore, oleic acid molecules possess only one double bond in the aliphatic chain has stronger hydrophobicity property, hence they are less soluble



**Figure 5.** (a) Mean particle size and (b) mean zeta potential of (■) oleate-oleic acid, (●) linoleate-linoleic acid and (▲) linolenate-linolenic acid as a function of incubation time in 50 mM aqueous borate buffer solution at pH 9.0 at room temperature (28 °C). Opened symbol indicates the respective fatty acid with DPPE-PEG2000.

in aqueous solution. This further explains why oleate-oleic acid solution indicated the lowest CVC value. On the contrary, linolenate-linolenic solution that corresponds to three unsaturations has the highest CVC value owing to the fact that it has the weakest hydrophobicity property. The similar results were observed for vesicles with incorporation of DPPE-PEG2000. However, CVC values are higher with the present of DPPE-PEG2000 compared to those without DPPE-PEG2000. This is due to the present of DPPE-PEG2000 as anionic molecules in the solution increase the ratio of ionized to non-ionized molecules and hinder the formation of vesicle. Therefore, the higher amounts of fatty acid molecules are required to uphold the ratio. It can be achieved by increase the concentration of fatty acid meanwhile pH of the solution is maintained.

**Particle Size and Zeta Potential of Vesicles.** Particle size of a fatty acid vesicle is affected by the fatty acid chain length and degree of unsaturation. Fatty acid with shorter chain length tends to form a larger vesicle than the longer chain fatty acid. This may be due to shorter aliphatic chain length is more rigid, consequently forming larger vesicle with less curvature. With

**Table 2.** Loading efficiency of fatty acids' vesicle

Fatty acid	Loading efficiency, %	
	Calcein	DL- $\alpha$ -tocopherol acetate
Oleate-oleic acid	4.4	61.2
DPPE-PEG2000 -oleate-oleic acid	3.1	47.2
Linoleate-linoleic acid	2.3	44.1
DPPE-PEG2000-linoleate-linoleic acid	2.0	39.0
Linolenate-linolenic acid	2.3	44.8
DPPE-PEG2000-linolenate-linolenic acid	2.2	32.3

this idea in mind, it is expected that the higher the number of unsaturation in the hydrocarbon chain, the smaller the vesicle will be formed. The plausible explanation could be owing to the nature of intermolecular interaction that caused more stacking in the membrane as the number of unsaturation is less, thus leading to formation of "flatter" bilayer. Therefore, by increasing the degree of unsaturation in the lipid acyl chain, the more bends and kinks are present and hence membrane fluidity will also be increased that resulting in the formation of vesicle with higher curvature or smaller in particle size.<sup>15-17</sup>

The mean particle size and zeta potentials of fatty acid vesicle solutions with and without DPPE-PEG2000 were monitored for a period of 30 days at fatty acid concentration of 5 mM which is well above their CVC values as presented in Figure 5a. The particle size of all three types unsaturated fatty acid vesicles without DPPE-PEG2000 are larger than 100 nm as a result of polydispersity nature of the vesicle dispersion with polydispersity index values ranging from 0.1 - 0.6. The mean size for oleate-oleic acid vesicles found to be the largest whilst linolenate-linolenic acid vesicles showed markedly smaller than the others. By comparing the three fatty acids, oleic acid with only one unsaturation is considered to be less flexible, higher stacking and less curvature leading to larger vesicles. On the other hand, molecules with two or more saturations are more flexible and therefore have higher lateral diffusion coefficient than the monounsaturations. The dynamics nature of the monomers in the bilayer membrane and more fluidic nature of the bilayer for higher unsaturation lead to higher membrane stability and curvature. This explains linolenate-linolenic suspension was dominated with smaller size vesicles. Nevertheless, the particle size of linoleate-linoleic acid and linolenate-linolenic acid vesicles were increased drastically after 7 days of storage at room temperature (28 °C) as a result of aggregation. However, there is no apparent change in the size of the DPPE-PEG2000-oleate-oleic acid vesicle solutions which indicates the stability of the vesicles. Although lots of works had been reported on improving the stability of phospholipid vesicles through incorporation of stealth into the vesicles, unfortunately, it was not totally applicable on fatty acid vesicles as revealed from our finding. Obviously, Figure 5b demonstrates that only oleate-oleic acid vesicles promote significantly reduce in zeta potential to a less negative value after the incorporation of DPPE-PEG2000 to the vesicles. This is probably due to the long and bulky polyoxyethylene group wrapping around the vesicles that reduced mobility of vesicles, and hence the zeta potential. This observa-

tion also implicates the interaction form between the oleate-oleic acid and DPPE-PEG2000 is the strongest among the acids. In contrast, vesicles forming from linoleic acid and linolenic acid with the incorporation of DPPE-PEG2000 did not display any significant change on the zeta potential. In other words, DPPE-PEG2000 is unlikely to interact with linoleic acid and linolenic acid in the formation of vesicles at pH 9.0. Therefore, these vesicles were not as stable as in the case of oleate-oleic vesicles. Hence, explanation for the increase in zeta potential to a less negative value for linoleate-linoleic acid vesicles and linolenate-linolenic acid vesicles after day seven was due to aggregation effect. These results are in agreement with the increase of vesicle size after day seven.

#### Loading Efficiency of Calcein and DL- $\alpha$ -Tocopherol acetate.

There are several factors affect the loading efficiency of vesicles, which include the concentrations and chemical properties of corresponding substances and also the preparation method.<sup>18,19</sup> The loading efficiencies of calcein and DL- $\alpha$ -tocopherol acetate on C<sub>18</sub> unsaturated fatty acid vesicles were calculated by applying Eq. 1 and are listed in Table 2. In general, all vesicles prepared *via* current method show different capability in encapsulation of the above mentioned substances. It is also observed that DL- $\alpha$ -tocopherol acetate associated with remarkably higher loading efficiency under our working conditions. The plausible explanations are DL- $\alpha$ -tocopherol acetate that behaves as a hydrophobic substance tends to be embedded in between the bilayer. On the other hand, the loading efficiency of calcein encountered in this study is considerably low (2 to 4%) regardless of the vesicle size. Yet, as revealed by other researchers, the calcein loading efficiencies may vary from 0.1% to 39.5% subjected to the factors mentioned above.<sup>8, 19-22</sup>

#### Conclusion

The present work reports the effect of vesicle stability as a function of unsaturation degree in fatty acid molecules. The membrane become more fluid liked as the degree of unsaturation increase, therefore thickness of membrane will be reduced. As a consequence the membrane will become more flexible. Thus, the particle size of the vesicles is expected to be smaller. It has also been observed that vesicles prepared from mono-unsaturated fatty acid are the most stable suspension for at least 30 days compared to fatty acid with two saturations and three saturations. Lastly, the incorporation of DPPE-PEG2000 does not always exhibit its ability on improving the stability of vesicles as revealed in this study.

**Acknowledgments.** This research work was supported by the Ministry of Science, Technology and Innovation Malaysia (MOSTI) under the eScienceFund 03-01-03-SF0316 and Postgraduate Research Grant (PPP) P0267-2007A from University Malaya.

#### References

- Morigaki, K.; Walde, P.; Misran, M.; Robinson, B. H. *Colloids Surf. A: Physicochem. Eng. Aspects.* **2003**, *213*, 37.
- Boris, B. N.; Tiong, N. S.; Misran, M. *Coll. Surf. A: Physicochem.*

- Eng. Aspects* **2004**, 236, 7.
- Bangham, A. D.; Sandish, M. M.; Watkins, J. C. *J. Mol. Biol.* **1965**, 13, 239.
  - Gebicki, J. M.; Hicks, M. *Nature* **1973**, 243, 232.
  - Hargreaves, J. W.; Deamer, D. W. *Biochemistry* **1978**, 17, 3759.
  - Gebicki, J. M.; Hicks, M. *Chem. Phys. Lipids* **1976**, 16, 142.
  - Rogerson, M. L.; Robinson, B. H.; Bucak, S.; Walde, P. *Coll. Surf. B: Biointerfaces* **2006**, 48, 24.
  - Namani, T.; Ishikawa, T.; Morigaki, K.; Walde, P. *Coll. Surf. B: Biointerfaces* **2007**, 54, 118.
  - Albena, N. K.; Malcom, N. J. *Biochimica et Biophysica Acta* **1996**, 1304, 120.
  - Minakshi, G.; Tathagata, D.; Narendra, K. J. *AAPS PharmaSciTech* **2007**, 8(2), E1.
  - Klibanov, A. L.; Maruyama, K.; Torchillin, V. P.; Huang, L. *FEBS Lett.* **1990**, 268, 235.
  - Ishii, F.; Nagasaka, Y. *Journal of Dispersion Science and Technology* **2001**, 22, 97.
  - Nii, T.; Takamura, A.; Mohri, K.; Ishii, F. *Colloids and Surfaces B: Biointerfaces* **2002**, 27, 323.
  - Kanicky, J. R.; Dinesh, O. S. *J. Colloid and Interface Science* **2002**, 256, 201.
  - Cevc, G. *Biochemistry* **1991**, 30, 7186.
  - Keough, K. M.; Giffin, B.; Kariel, N. *Biochim. Biophys. Acta* **1987**, 902(1), 1.
  - Stubbs, C. D.; Kouyama, T.; Kinosita, K.; Ikegami, A. *Biochemistry* **1981**, 20, 4257.
  - Liu, N.; Park, H. J. *Colloids and Surfaces B: Biointerfaces* **2010**, 76, 16-19.
  - Bahiaa, C. O. A. P.; Azevedoa, E. G.; Ferreirab, A. M. L.; Frézarda, F. *European Journal of Pharmaceutical Sciences* **2010**, 39, 90-96.
  - Memoli, A.; Palermi, G. L.; Travagli, V.; Alhaique, F. *J. Soc. Cosme. Chem.* **1994**, 45, 167-172.
  - Manosroi, A.; Wongtrakul, P.; Manosroi, J.; Sakai, H.; Sugawara, F.; Yuasa, M.; A. M. *Colloids and Surfaces B: Biointerfaces* **2003**, 30, 129-138.
  - Fan, X. J.; Liu, Q.; Zhen, P.; Zhang, Y.; Hu, X. *Journal of Chinese Pharmaceutical Sciences* **2007**, 16, 96-100.
-