

## Effect of pH on Physicochemical Properties and Encapsulation Efficiency of PEGylated Linolenic Acid Vesicles

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**Abstract:** The preparation of vesicle from a mixture of linolenic acid and 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine-N-[methoxy-(polyethylene glycol)-2000] (DPPE-PEG2000) has been successfully carried out by using dry lipid hydration method. The effect of pH on particle size, zeta potential, encapsulation efficiency and critical vesiculation concentration (CVC) of the prepared vesicle suspensions in aqueous were studied. Macroscopic stability of the vesicles was also evaluated through their particle size and zeta potential for a period of 30 days. We found that CVC vary according to the pH, with higher pH of the bulk solution, CVC is higher. Vesicles formed at pH 8.5 were the most stable suspension throughout a period of 30 days compared to those at pH 7.5 and pH 9.0. Addition of DPPE-PEG2000 into the preparation of vesicle at pH 8.5 caused a reduction of the vesicle size to the scale of nanometer which is an advantage to their application. On the other hand, encapsulation of calcein and vitamin E were carried out. Certain amount of these compounds could be successfully loaded into the resulting liposomes under this experimental condition.

**Keywords:** linolenic acid, Vesicle, pH, DPPE-PEG2000.

### Introduction

Vesicles are bilayer spherical aggregates of amphiphilic molecules with the basic matters are commonly consisting of a hydrophilic head group and double hydrocarbon tailed such as phospholipid. Vesicles have long been used as a tool for the delivery of vaccines, enzymes and drugs in the body because it can direct a drug to the target. At the same time vesicles also act as a reservoir in release of the drug at a slower rate. Therefore healthy cells are shielded from the drug's toxicity, especially at the vulnerable tissues such as kidneys and liver. In addition, vesicles are biocompatible, low risk of toxicity, commercially available and the preparation methods are not too complicated<sup>1</sup>.

Amphiphiles with single hydrocarbon tail such as fatty acids have also been used to prepared vesicles. Oleate-oleic acid vesicles were the first single chain fatty acid vesicle

successfully prepared by Gebicki and Hicks in 1973<sup>2,3</sup>. Later, vesicles were also prepared from polyunsaturated fatty acid such as linoleic acid (*cis,cis*-9,12-octadecadienoic acid)<sup>4,5</sup> and *cis*-4,7,10,13,16,19-docosahexaenoic acid<sup>6</sup>. Besides, short chain saturated fatty acids such as octanoic acid and decanoic acid<sup>7</sup> have also been reported to form vesicles successfully.

Fatty acids are selected instead of phospholipids because they are less expensive, easily hydrolyzed, simple molecular structure and present in the membrane naturally. However, the formation of fatty acid vesicles is restricted to certain range of pH, type of ionic composition in the buffer solution and concentration of the fatty acid<sup>8</sup>. For a solution at pH approximately to the pKa of the fatty acid, concentration ratio of the ionized to non-ionized fatty acid molecules are  $\approx 1$ . Hence, pseudo-double-chain surfactant can be formed through hydrogen bonding where a proton from the non-ionized carboxylic acid is shared by the adjacent ionized carboxylated molecule that has the appropriate geometry to induce the formation of bilayer and hence vesicle.

Nevertheless, fatty acid vesicles encounter a challenge like other colloidal system which is stability. They are facing trouble on retain their physical particle size with no agglomeration or flocculation during time of storage. Environment of the fatty acid dispersing medium such as pH and ionic concentration play an important role in maintaining the stability of vesicle suspension. Hence, lots of works have been carried out in order to improve the stability of vesicles suspension.

One of the successful works that enhanced the stability of fatty acid vesicle was by extending the pH range of vesicle formation. In order to form vesicle in a more acidic region, addition of amphiphilic molecule with headgroup of sulphonate, sulphate or oligo(ethylene oxide) unit intercalated between the hydrocarbon chain and the carboxylate were found to be effective<sup>9</sup>. On the other hand, addition of long chain linear alcohol has successfully shifted the pH for vesicle formation towards alkaline region<sup>10</sup>. To our knowledge, no literature work has been reported regarding the effect of pH on PEGylated fatty acid vesicle solution. Although fatty acid vesicles can be formed in a narrow range of pH, evaluation on the physicochemical properties of the vesicles within that range of pH are still not clear. It is vital to open a new path way of application with additional information about the changes occurred as the pH change slightly although still within the pH range for vesicle formation. Another alternative that has been proven to enhance the stability of the vesicle is through steric stabilization by modifying the surface of the vesicle<sup>11-13</sup>. In this regard, a bulky hydrophilic head group is introduced to the vesicle. This can be done by incorporation of either natural or synthetic substances such as glycolipid, glycoprotein, polysaccharides, lectins and synthetic polymer. Nevertheless, less attention has been paid to the relevancy of synthetic polymer in stabilization of the fatty acid vesicle.

In the present study, investigation of pH effect on the stability of linolenate-linolenic acid vesicle was carried out through evaluation of the data from particle size and zeta potential over a storage period of 30 days. Furthermore, synthetic polymer covalently bonded to phospholipid; 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoethanolamine-*N*-[methoxy (polyethylene glycol)-2000] (DPPE-PEG2000) was added to the formation of vesicle and the effect of this substance with respect to pH on the physicochemical properties of vesicle was also studied. The efficiency of vesicle to encapsulate water soluble and water insoluble materials will also be explored.

## Experimental

Alpha-linolenic acid (*cis,cis,cis*- 9, 12, 15- octadecatrienoic acid) was from Sigma (St. Louis, USA) with purity  $\geq 99.0\%$ , 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoethanol-

amine-N-[methoxy(polyethylene glycol)-2000] (DPPE-PEG2000) sodium salt was purchased from Avanti polar lipids (Alabaster, AL), boric acid with minimum 99.5% was from Fluka (Buchs, Switzerland), hydrochloric acid, sodium hydroxide 98 % and chloroform (distilled) were from HMBG Chemicals, sodium dihydrogen phosphate dehydrate  $\geq 99.0\%$  and disodium hydrogen phosphate  $\geq 98\%$ , DL- $\alpha$ -tocopherol acetate  $\sim 98\%$  and calcein  $\geq 93\%$  were from Fluka. All chemicals were used as received. Deionized water with resistivity  $18.2 \text{ M}\Omega \text{ cm}$  was obtained from Barnstead NANOpure<sup>®</sup> Diamond<sup>™</sup> ultrapure water system. Deionised water was doubly distilled and deaerated with dried nitrogen gas prior to use.

## **Investigation of the effect of pH on vesicles' size and zeta potential**

### *Preparation of stock solution*

A stock solution of 12.5 mM alpha-linolenic acid in 27.5 mM NaOH was prepared by mixing both chemicals. The solution was stirred until a transparent solution was obtained. Stock solution of alpha-linolenic acid containing DPPE-PEG2000 was prepared by first dissolving 12.5 mM alpha-linolenic acid and 0.25 mM DPPE-PEG2000 in 2 ml Chloroform. The mixture was then placed into a rotary evaporator at 40 °C to remove all of the chloroform. Warm distilled water at 50 °C and 27.5 mM NaOH solution were slowly added. The solution was then sonicated for 5 minutes.

### *Acid-base titration of the stock solution with HCl (0.125 M)*

A stock solution of 1.500 mL was pipetted into a 14.5 ml sample vial followed by addition of  $(1.500 - x)$  mL deionised water and  $x$  mL HCl (0.125 M). The solution was mixed for 1 minute by vortex mixer Uzusio VTX 3000L then the pH of the solution was measured by a Mettler Toledo pH meter. Calibration was performed at the titration temperature with buffer of pH 4.0, 7.0 and 9.2. An average of 3 measurements was carried out. A similar procedure was repeated for alpha-linolenic acid containing DPPE-PEG2000.

### *Light microscopy observation*

The formations of vesicle in the solutions were observed with LEICA DMRXP Germany made light polarizing microscope. Images were focused by bright field and dark field mode between cross polarizers using oil immersion technique. This system consists of a high voltage beam source, a polarizing unit and a detector unit. The detector unit is interface with a personal computer equipped with image analysis software (Leica Qwin Standard version 2.6) that helps capture and import images from the microscope.

### *Transmission electron microscopy*

The vesicle images at 40 mM were obtained by using Hitachi H-7100 transmission electron microscope with the negative-staining method. The sample was prepared by immersed the formvar-coated copper grid into a drop of the vesicle solution and allowed to stand for 10 minutes. The excess vesicle solution was blotted with filter paper and followed by staining process with 3% 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 100 kV.

### *Particle size and zeta potential measurement*

The hydrodynamic size and zeta potential of the vesicles with various pHs were determined by dynamic light scattering (DLS) Malvern particle size analyzer, Model Nano ZS (Worcestershire, UK). All particle size and zeta potential measurements were made at the scattering angle of 175° and 17°, respectively. The measurements were carried out at

temperature of 30 °C. The mean value is obtained from three measurements. Similar method was applied on the evaluation of vesicles' stability.

#### *Determinations of CVCs for alpha-linolenic acid solutions*

A series of solutions with various concentration of alpha-linolenic acid at pH 7.5, pH 8.5 and pH 9.0 were prepared. Borate buffer pH 8.5 or pH 9.0 of 50 mM after make up to the volume was added to the solution at desired pH. Similarly, 50 mM of phosphate buffer pH 7.5 was used to prepare vesicle solution of pH 7.5. The solutions were then filtered by use of a 0.45 µm nylon filter from (Minisart®) prior to measurements. PEGylated vesicle solutions of alpha-linolenic acid containing DPPE-PEG2000 were prepared by the similar procedure. The CVC determinations were carried out at 30 °C employing du Nouÿ ring tensiometer balance from KRUSS with K12 tensiometer processor.

#### *Evaluation of stability*

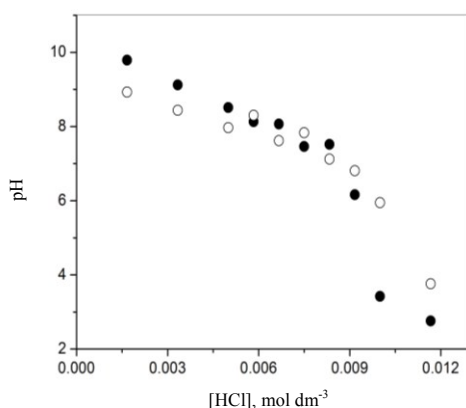
Vesicle solutions with concentration of 2 mM at pH 7.5, pH 8.5 and pH 9.0 were prepared as mentioned in the determination of CVC and stored at 28 °C for a period of 30 days. Measurement of particle size and zeta potential for the vesicle solutions were carried out by using the similar method as stated above during that period of time.

#### *Encapsulation of calcein and vitamin E*

A mixture of 25 mM fatty acid and 0.5 mM calcein in chloroform was dried under a rotor evaporator at 40 °C. Similarly, the PEGylated vesicle was prepared in the same manner but this time 0.5 mM of DPPE-PEG2000 was added. Warmed deionized water at 50 °C was then slowly added to the dried lipid followed by addition of buffer solution at the desired pH with make up final concentration of 50 mM. The solution was then placed in a bath sonicator (JAC Ultrasonic 1505) at 30 °C for 5 minutes. Similar method was applied for the encapsulation of vitamin E. The encapsulated and non-encapsulated substances were separated by using mini-column method<sup>14</sup>. Pretreatment of Sepharose 4B with mobile phase for swelling purpose was used as a stationery phase. A small piece of cotton was placed at the bottom of the barrel of a 5 mL plastic syringe prior to packing with Sepharose 4B in order to prevent leakage of sepharose 4B. Then the column was inserted into a centrifugal tube so that it was securely supported at the top of the test tube by the finger grips of the syringe. A volume of 50 µL vesicle solution containing entrapped and free solute was applied to the Sepharose 4B bed followed by 3 × 0.5 mL of mobile phase. The column was spun at knob 4 for 2 minutes in an MSE centrifuge with swinging buckets. The first separation that comes out into the centrifugal tube was the encapsulated vesicle solution that latter was diluted to 5 mL with distilled ethanol. The remaining solute retained by the Sepharose was recovered by washing the column with the mobile phase and eluted by centrifugation at the similar speed for three times followed by dilution to 10 mL with distilled ethanol. Both solutions of encapsulated and non-encapsulated vitamin E were then analyzed using Shimadzu LC-20AT High Performance Liquid Chromatography coupled with SPD-20A prominence UV/Vis Detector. The isocratic mobile phase system composed mixture of all HPLC grade solvents; ethanol/methanol/acetonitrile (45:45:10 by volume) was delivered to the column at a flow rate of 1 mL min<sup>-1</sup>. A sample of 20 µL was injected into the C18 reverse phase Purospher® Star column with dimension 4.6 mm × 250 mm and the particle diameter of 5 µm. The eluent was monitored at 287 nm, and the detector temperature was set at 25 °C. Analysis of calcein concentration was carried out by Cary 50 UV-Vis Spectrophotometer at 496 nm. The encapsulation efficiency was calculated from the percentage value of absorbance for encapsulated calcein at 496 nm or the area under peak at retention time 9.2 min for encapsulated vitamin E over the absorbance or area for total amount of solute.

## Results and Discussion

The equilibrium titration curve of linolenic acid and PEGylated-linolenic acid as a function concentration HCl added is illustrated in Figure 1. A transparent solution composes of all ionized fatty acid molecules were observed at pH above 9.5. Further addition of HCl into the alkaline solution resulted in protonation of some anionic linolenate molecules. Hence, addition of HCl may increases the amount of non-ionized fatty acid molecules. The coexistence of ionized and non-ionized molecules in the solution leads to the formation of pseudo-double-chain surfactant between the head groups of  $\text{COO}^-$  and  $\text{COOH}$  via hydrogen bond. From our studies, linolenic acid is found to form vesicles in the pH range of solution from 7.5 to 9.0. As can be seen from figure 1, the pH of mixed fatty acid and DPPE-PEG2000 is lower compared to solution with only fatty acid. The presence of DPPE-PEG2000 with a long hydrophilic polyethylene glycol head group moiety would coat around the surface of spherical vesicle and hence promotes steric stabilization to the vesicles. It has also been agreed that PEG promotes the formation of vesicle and inhibits the aggregation of the vesicle through the long ethoxylate hydrophilic chains that extended out to at least 5 nm from the surface of bilayer<sup>15,16</sup>. This hydrophilic chain also plays a role in shielding the ionized fatty acid molecules from react with proton. As a result, concentration of free proton in the bulk solution is normally higher, therefore the pH is lowered.



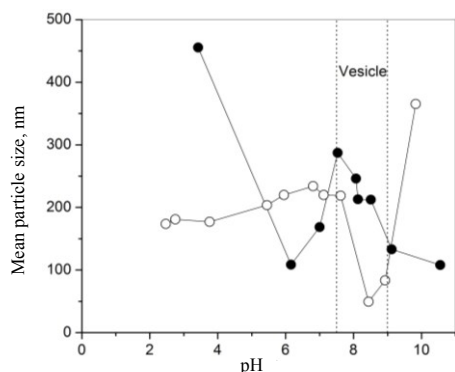
**Figure 1.** Equilibrium titration curve of linolenic acid (●) 12.5 mM and PEGylated linolenic acid (○) 12.5 mM at 28 °C as a function concentration HCl.

The mean particle size and zeta potential for both linolenate-linolenic acid vesicles and PEGylated linolenate-linolenic acid vesicles at pH range from 3.5 to 10.0 were displayed in figure 2 and figure 3, respectively. Both of the figures show a drastic change in particle size and zeta potential values at the pH region about 7.5 to 9.0. The addition of DPPE-PEG2000 into linolenic acid promotes the formation of smaller size vesicles. Furthermore, the magnitude of zeta potential for mixture of DPPE-PEG2000-linolenate-linolenic acid vesicles was also significantly smaller than those of linolenate-linolenic acid vesicle solution.

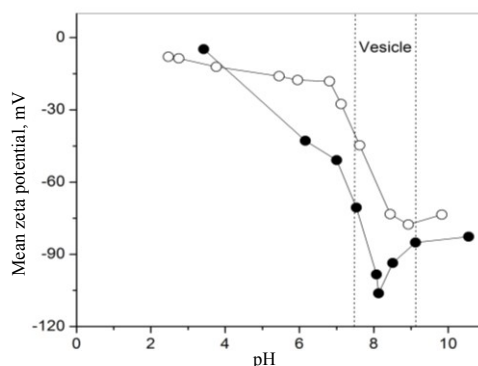
A plausible explanation for the formation of smaller size vesicle with incorporation of DPPE-PEG2000 is due to the presence of long PEG chain that is more dynamic in an aqueous solution<sup>17</sup>. As a result, the effective head group area is larger with addition of DPPE-PEG2000 that lead to a smaller value of packing parameter compared to the packing parameter in vesicle composing only linolenate-linolenic acid. A smaller value of packing parameter that still within the value greater than 0.5 but smaller than 1 may favors the formation of particles with higher curvature which promote the molecules to pack tightly.

Therefore, particle size for PEGylated linolenate-linolenic acid vesicle is smaller compared to linolenate-linolenic acid vesicles. This observation supports the theory of successful insertion of DPPE-PEG2000 into the bilayer of vesicle.

A further confirmation on insertion of DPPE-PEG2000 into the membrane bilayer was revealed from the measurement of zeta potential as indicated in Figure 3. The decrease in magnitude of zeta potential is due to the presence of long ethoxylate polymer chain in DPPE-PEG2000 that coated around the surface of vesicles and hidden the negatively charge underneath the polymer layer. As a consequence, the mobility of the vesicles was reduced and the zeta potential becomes less negative.

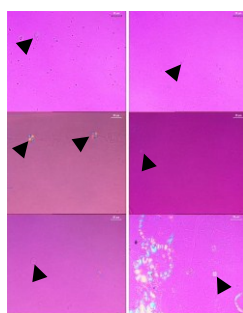


**Figure 2.** Mean particle size of 12.5 mM linolenic acid (●) and 12.5 mM PEGylated linolenic acid (○) at various pH.



**Figure 3.** Mean zeta potential of 12.5 mM linolenic acid (●) and 12.5 mM PEGylated linolenic acid (○) at various pH.

The presence of vesicle was authenticated by appearance of cross-maltese images that showing the lamellar structure of the spherical vesicle suspension using light polarizing micrograph. In fact, Figure 4 confirmed the formation of vesicles at pH 7.5, 8.5 and 9.0.



**Figure 4.** Polarizing micrograph of (1) linolenate-linolenic acid vesicle and (2) PEGylated linolenate-linolenic acid vesicle at pH (A) 7.5, (B) 8.5 and (C) 9.0.

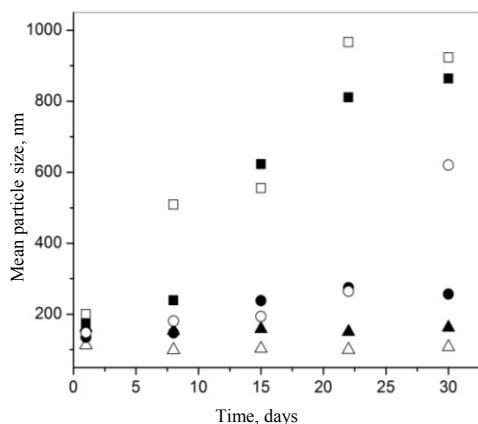
In this regard, the minimal concentration of linolenic acid required to form stable membrane bilayer which is known as critical vesiculation concentration (CVC) was also determined via surface tension method. As indicated in Table 1, CVC of linolenic acid and PEGylated linolenic acid at pH 7.5 is the lowest with assumption that emulsion was not

formed. The formation of fatty acid vesicle is dominated as the ionized carboxylate and non-ionized carboxylic are co-exist. As mentioned elsewhere, pKa of linolenic acid is 8.28<sup>18</sup>. Therefore, at pH approximately 8.28, concentration of linolenate and linolenic acid are almost equimolar and the formations of pseudo-double-chain surfactant via hydrogen bonds that can be analogue to phospholipid are encouraged. Thus, it leads to engender of bilayer vesicle. However, at the pH higher than pKa, CVC of the solution is the highest due to considerable increase amount of linolenate monomer in the solution. Thus at pH 9.0, the overall hydrophilicity in the solution is higher that results in a weaker driving force for self aggregation. As a consequence, higher amount of non-ionized linolenic acid is required and resulting in a higher CVC. In contrary, despite the amount of linolenate anion is less at pH 7.5, the concentration of linolenic acid is sufficient for vesicle formation through the hydrophobic interactions and hydrogen bonds. Similar trend was found for linolenic acid with incorporation of DPPE-PEG2000 where we observed CVC raised as pH increased. Yet, CVC for the solution of linolenate-linolenic acid with incorporation of DPPE-PEG2000 is found significantly different compared to the pure linolenate-linolenic acid solution. The result indicates that at pH 8.5 and pH 9.0, addition of DPPE-PEG2000 to linolenic acid increased the CVCs. In general, DPPE-PEG2000 with negatively charge causes an additional concentration of anion in the solution. Therefore, at pH 8.5 and pH 9.0, concentration of anion in the solution is higher compared to the non-ionized linolenic acid. In order to achieve the formation of vesicle, much more non-ionized linolenic acid molecule is required and this leads to an increase in CVC. In other words, addition of DPPE-PEG2000 destabilized the formation of bilayer at pH 8.5 and pH 9.0. In contrast, CVC for linolenic acid-DPPE-PEG2000 solution at pH 7.5 is lower than CVC of linolenic acid. This can be explained by lower amount of linolenate anion is required due to the availability of anionic DPPE-PEG2000. The results obtained here are consistent with the findings reported by Charles *et. al.*. They found that formation of nonanoate-nonanoic acid vesicle was stabilized by addition of nonanol and reported a lower CVC (20 mM) compared to vesicle prepared from only nonanoic acid (85 mM)<sup>10</sup>.

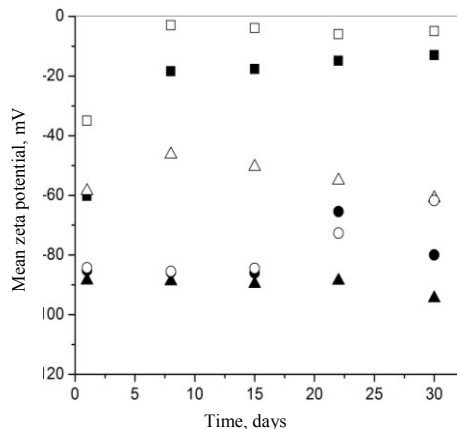
**Table 1.** Critical vesiculation concentration (CVC) of linolenate-linolenic acid vesicle and PEGylated linolenate-linolenic vesicle at various pH.

pH	Critical vesiculation concentration, mM	
	Linolenic acid	DPPE-PEG2000-linolenic acid
7.5	0.24	0.15
8.5	0.88	0.91
9.0	1.23	1.48

A colloidal system is considered stable if the particles do not aggregate into clusters at a significant rate. As illustrated from Figure 5, mean particle size of linolenate-linolenic acid vesicles and vesicles consist of DPPE-PEG2000 at pH 7.5 increase gradually. The plausible explanation for this is due to the hydrophobic nature of the non-ionized linolenic acid. In contrast, it is observed from figure 6 that zeta potential becomes less negative for PEGylated vesicle with time. This phenomenon implicates the aggregation of vesicles which lead to slower mobility and thus the magnitude of zeta potential. Similarly, vesicles formed at pH 9.0 from both pure linolenic acid and PEGylated lipid mixed with linolenic acid only manage to maintain their particle size for about 7 days. This is also revealed by a decrease in magnitude of the zeta potential. An important feature discovered from these results is DPPE-PEG2000 did not play a significant role in stabilization of the vesicle suspension at pH 7.5 and pH 9.0 as expected.

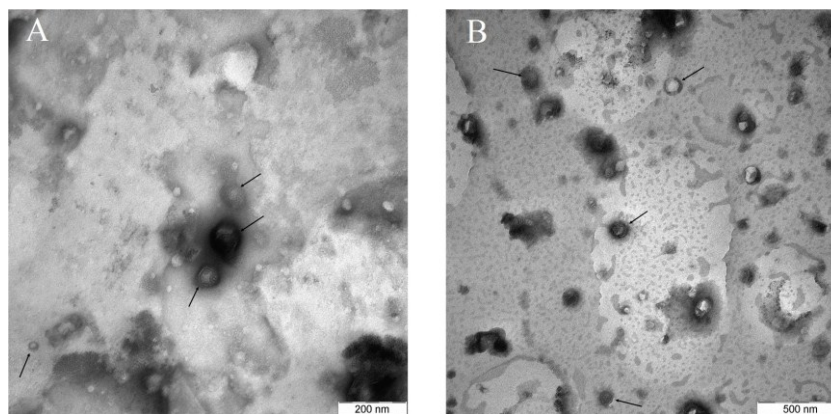


**Figure 5.** Mean particle size at pH 7.5 (■, □), 8.5 (▲, △) and 9 (●, ○) of linolenate-linolenic acid vesicle (filled symbol) and PEGylated linolenate-linolenic acid vesicle (empty symbol).



**Figure 6.** Mean zeta potential at pH 7.5 (■, □), 8.5 (▲, △) and 9 (●, ○) of linolenate-linolenic acid vesicle (filled symbol) and PEGylated linolenate-linolenic acid vesicle (empty symbol).

On the other hand, our results suggested that the most stable vesicle suspension can be formed at pH 8.5 as their mean vesicle size and zeta potential were almost consistent regardless the incorporation of DPPE-PEG2000. Nevertheless, it is observed that vesicles incorporated with DPPE-PEG2000 were smaller with particle size around 100 nm at pH 8.5. This might be due to the presence of bulky hydrophilic PEG chain at the bilayer surface promoting the molecules to pack tightly with higher curvature. Therefore, this has proven the effectiveness of DPPE-PEG2000 participates in the formation of vesicles as to enhance the stabilization of vesicle suspension in an aqueous solution. An additional transmission electron micrograph for linolenic acid and PEGylated linolenic acid at pH 8.5 were shown in Figure 7.



**Figure 7.** TEM micrograph of 40 mM linolenate-linolenic acid vesicle (A) and PEGylated linolenate-linolenic acid vesicle (B) at pH 8.5. The presences of vesicles are indicated by the arrow.



Since the stability of the vesicle has been identified, encapsulation of water soluble and water insoluble materials such as calcein for former and vitamin E for latter were also studied. From table 2, it is found that the encapsulation efficiency of vitamin E in the vesicle is at least 10-fold higher than the encapsulation efficiency of calcein under our experimental condition. One of the plausible explanations for this observation is due to hydrophobicity nature of vitamin E which shows higher preferences to be embedded in the membrane bilayer. In contrary, calcein is water soluble especially at high pH, so they have a higher tendency of escaping in to the bulk solution during the process of bilayer convolution. As a result, the entrapped calcein that attribute to the percent of loading may be either from calcein entrapped at the hydrophilic layer of the membrane or those within the multilamellar structure. This explains the low percentage of calcein encapsulation by the vesicles. Similar reason also applicable to the vesicles with insertion of DPPE-PEG2000.

As the pH increase, the encapsulation of vitamin E decrease. This could be due to higher surface polarity and stronger electron density at the head group that inhibit permeation of vitamin E into the membrane. On the other hand, the encapsulation efficiency of vitamin E in PEGylated vesicle was found lower than linolenic acid that probably due to their smaller particle size. In addition, another possible explanation is the number of lamellae is reduced with incorporation of PEG as reported by Belsito *et al.*<sup>19</sup>. Therefore, limited amount of vitamin E can be loaded into to bilayer.

**Table 2.** Percentage encapsulation efficiency of linolenate-linolenic acid vesicle and PEGylated linolenate-linolenic acid vesicle at pH 7.5, 8.5 and 9.0.

pH	% Encapsulation Efficiency			
	Vitamin E		Calcein	
	Linolenate-linolenic acid vesicle	PEGylated linolenate-linolenic acid vesicle	Linolenate-linolenic acid vesicle	PEGylated linolenate-linolenic acid vesicle
7.5	83±5	65±5	0.3±0.1	0.9±0.4
8.5	55±6	39±6	3.4±0.5	3.0±0.8
9.0	45±4	32±6	2.3±0.3	2.5±0.9

## Conclusion

Physico-chemical properties of linolenate-linolenic acid vesicle can be altered by pH and/or addition of DPPE-PEG2000. Although vesicles can only be formed at a narrow range of pH as reported elsewhere, their physico-chemical properties deviate significantly in that region. In general, CVC vary according to the pH, higher pH associated with higher CVC and vice versa. In this study, vesicles formed at pH 8.5 are the most stable suspension regardless the incorporation of DPPE-PEG2000. The incorporation of DPPE-PEG2000 in the preparation of vesicles at pH 8.5 promotes the formation of nano size particles that broaden their field of application. Moreover, the encapsulation efficiencies of calcein and vitamin E display encouraging values.

## Acknowledgment

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