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INTRODUCTION

Hyaluronic acid (HA), also called hyaluronan, is a natural glycoaminoglycan (polysaccharide) that is mostly abundant in skin [1]. However, as skin ages, the hyaluronic acid in the skin decreases which accounts for the loss of hydration and moisture in the skin and ultimately results in wrinkle and loss of elasticity. As a consequence, HA is extensively utilized in cosmetic products to combat signs of aging [2]. Nevertheless, owing to its high molecular weight and charge, it is difficult to deliver HA into the skin.

To overcome this problem, elastic liposome has been developed by reversed phase evaporation technique (REV). Elastic liposomes have been developed and evaluated as transdermal delivery systems to improve delivery of encapsulated agent to and through skin. They are similar to conventional liposomes but with the incorporation of an edge activator in the lipid bilayer structure to provide elasticity. The objective of this study was to develop and characterize the HA loaded liposome by varying the ratio of phospholipid (HPC): cholesterol (CH): Tween80. Additionally, the entrapment efficiency of HA loaded liposome was determined.

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MATERIALS AND METODS

Materials Hyarulonic acid sodium salt from *Streptococcus equi*, Stain All dye (assay 95%) and uranyl acetate dehydrate were purchased from Sigma Chemical, Capricorn, Singapore. Cholesterol from lanolin (≥ 95.0 % GC) was purchased from Sigma-Aldrich Chemie, Buchs, Japan. Chloroform and methanol (analytical grade) were purchased from RCI Labscan, Bangkok, Thailand. Ethanol 95% was obtained from commercial product, TTK-Science, Thailand. Phophatidylcholine (Lipoid S100-3) was purchased from Lipoid GmbH, Ludwigshafen, Germany and polyoxyethylene sorbitan monooleate (Tween 80) was purchased from Thai Sanguanwat Chemical, Bangkok, Thailand.

Determination of hyaluronic acid (HA) based on Stain All dye In this experiment, HA was determined based on Stain All by UV-visible spectrophotometer. Stain All is a dye which can bind to glycoaminoglycans and form complexes whose optical properties are different from those of the free dye. HA solution was prepared in deionized water with concentration ranging from 0.5-5 mg/ml. The dye solution (0.1 mg/ml) was prepared by dissolving 2.5 mg of Stain All in 22 ml of water and 3 ml of methanol. The HA/dye complex was detected at 640 nm [3].

Preparation of HA loaded liposome by reversed phase evaporation technique (REV) Hyaluronic acid loaded liposome was prepared by reversed phase evaporation technique. Phosphatidylcholine (HPC) and cholesterol (CH) were dissolved in a mixture of ethanol and chloroform (4:1). The aqueous phase, hyaluronic acid mixed with Tween 80, was added to the oil phase and mixed. The system was sonicated for 10 minutes in a bath type sonicator at 50°C for 10 minutes. Then, the organic solvent was removed under reduced pressure (200 mm Hg) using a rotary evaporator (Buchi Rotovapor, USA). At this point, the materials formed a liposomal suspension. The resulting liposomes were kept in the refrigerator overnight at -20°C before characterization [4].

Physicochemical characterization of the liposome Mean particle size: The mean particle size and size distribution measurements were conducted using a ZetaPALS® (Brookhaven Instrument Co., New York, USA). Prepared liposome formulations were diluted at least 50 times in DI water to obtain liposomal suspension. The particle size of each sample was measured at 25°C at a detection angle of 90° for 6 repeated measurements. All measures were performed in triplicate. Zeta potential: The zeta potential was determined using phase analysis light scattering with ZetaPAL/90plus. Measurements were carried out at 25°C at 14.8° to the incident light. Samples were prepared by redispersing the liposome in DI water. The zeta potential was calculated using the electrophoretic mobility based on the Smoluchowski approximation.

Morphology of liposome The morphology of the HA loaded liposome was characterized by transmission electron microscopy (TEM) with an accelerating voltage of 80 kV(Phillips Tecnai12, Electron Optics, Holland).The liposome specimen for TEM was prepared with negative staining. The prepared liposome

suspension was processed by using copper grid to adsorb liposome particles from the suspension for 5 min, then stained in uranyl acetate for 30 seconds and dried. The grid containing the liposome sample was dried further in the air and kept in desiccators before being investigated [5].

Determination of entrapment efficiency (EE): The entrapment efficiency of the HA loaded liposome was determined by ultracentrifugation method. Approximately 100 mg of HA loaded liposome was suspended in 2 ml deioinzed water and subjected to centrifugation at 18,000 rpm for 30 min. After discarding the supernatant, HA was extracted from liposome using 10 mL deionized water and sonication for 2 min at room temperature. Then, the sample was centrifuged at 18,000 rpm for 30 min. Finally, the supernatant was analyzed for HA content based on Stain All assay using a UV-Vis spectrophotometer at 640 nm. EE was calculated as [(Amount of HA entrapped) x 100]/(Total amount HA). The EE is reported as the mean of three independent trials.

RESULTS

Determination of HA based on Stain All dye

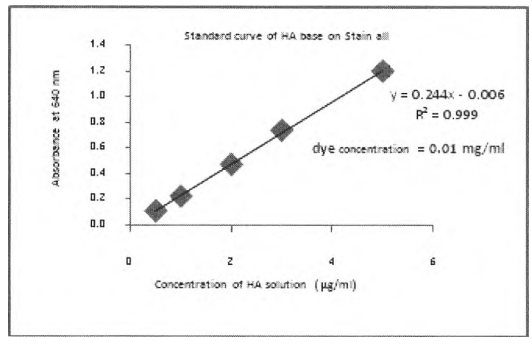


Figure 1 Standard curve of hyaluronic acid (HA) based on Stain All at 640 nm using UV-Vis spectrometer

Physiochemical characterization of elastic liposome

Table 1 Effect of HPC on the mean particle size, size distribution and zeta potential of elastic liposome

Code	HPC:CH:Tween80 (w/w)	MS (nm±SD)	PI (±SD)	BI (±SD)	Zeta potential (mV±SD)
A1	40:30:10	538 (±103)	0.18 (±0.09)	6.8 (±1.0)	-33.93 (±2.28)
A2	60:30:10	616 (±34)	0.06 (±0.07)	7.8 (±0.8)	-33.86 (±1.65)
A3	70:30:10	627 (±113)	0.07 (±0.06)	8.9 (±0.9)	-31.85 (±4.1)
A4	**80:30:10**	**577 (±36)**	**0.02 (±0.02)**	**8.4 (±0.9)**	**-26.45 (±2.46)**
A5	90:30:10	647 (±29)	0.01 (±0.01)	6.0 (±3.1)	-27.51 (±3.30)

* MS= mean particle size, PI =polydispersity index and BI =base line index

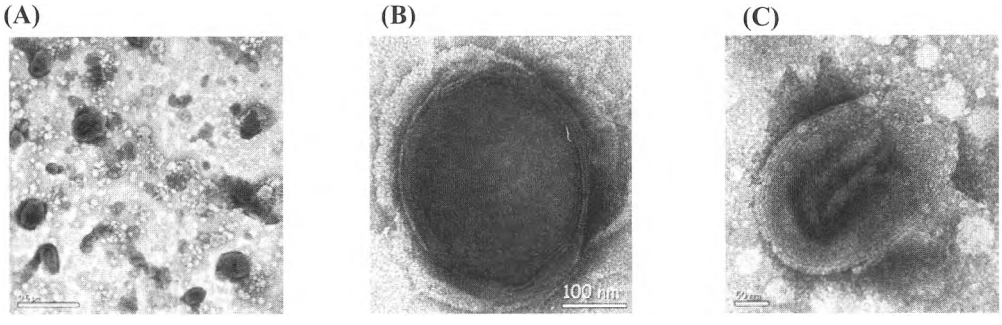
Table 2 Effect of CH on the mean particle size, size distribution and zeta potential of elastic liposome

Code	HPC:CH:Tween80 (w/w)	MS (nm±SD)	PI (±SD)	BI (±SD)	Zeta potential (mV±SD)
B1	80:0:10	N/A			
B2	80:10:10	773 (±57)	0.06 (±0.05)	3.7 (±1.7)	-22.05 (2.99)
B3	80:20:10	628 (±71)	0.007 (±0.03)	7.7 (±1.2)	-22.63 (2.23)
B4	80:30:10	522 (±36)	0.016 (±0.02)	6.8 (±8.1)	-25.02 (2.24)
B5	**80:40:10**	**539 (±18)**	**0.005 (±0.000)**	**8.1 (±0.7)**	**-24.19 (3.4)**

Table 3 Effect of Tween 80 on the mean particle size, size distribution and zeta potential of elastic liposome

Code	HPC:CH:Tween80 (w/w)	MS (nm±SD)	PI (±SD)	BI (±SD)	Zeta potential (mV±SD)
C1	80:40:5	527 (±102)	0.02 (±0.03)	8.7 (±0.6)	-25.93 (±2.24)
C2	80:40:10	539 (±18)	0.01 (±0.00)	8.1 (±0.7)	-24.19 (±3.4)
C3	80:40:20	446 (32)	0.01 (±0.00)	9.0 (±0.4)	-28.93 (±5.94)
C4	**80:40:30**	**382 (±32)**	**0.01 (±0.00)**	**9.0 (±0.4)**	**-28.21 (±6.37)**

Morphology of HA loaded liposome



Figures 2-4 The transmission electron micrographs in different fields of the HA loaded liposome prepared by REV method. TEM image (A):123000x, (B):135000x, and (C):135000x

Determination of entrapment efficiency

Table 4 The mean particle size, size distribution, zeta potential and % entrapment efficiency of HA loaded liposome

Concentration of HA (mg/ml)	MS (nm ±SD)	PI (±SD)	BI (±SD)	Zeta potential (mV±SD)	% Entrapment (±SD)	% drug content
0.2*	834 (±67)	0.06 (±0.07)	5.9 (±1.2)	-29.59 (±1.01)	74.90 (±3.13)	71.70
0.4*	515 (±44)	0.01 (±0.00)	8.1 (±0.9)	-25.88 (±1.8)	78.87 (±0.89)	78.98

*n=3

DISCUSSIONS

In this study, HA loaded elastic liposome was successfully developed. The effect of HPC: CH: Tween 80 on physical properties of elastic liposome was characterized. Table 1 shows that the mean particle size, polydispersity index and zeta potential are not significantly difference. In this experiment, increasing HPC resulted in increased liposome formation since, as a phospholipids, it could act in the formation of vesicle. Table 2 shows the effect of CH on physical properties of elastic liposome. The mean particle size tended to reduce when CH was increased because the insertion of CH molecules into the lipid bilayer helped the upper portions of the lipid hydrocarbon chains to adopt a more trans configuration, thus decreasing the number of kinks in the bilayer and increasing the orderliness of the lipid molecules. Thus, the inclusion of CH led to increased lateral packing density of phosphatidylcholine layers and decreased the free volume within the hydrophobic part of the layer lipid [6]. It is well known that CH acts as significant membrane stabilizer but also a relative vesicle formation. Thus, CH is indispensable component for liposome formation. Formulation without CH resulted in no liposome formation. Tween 80, as a non-ionic surfactant, acts as edge activator to enhance the skin permeation [7]. The effect of tween 80 on the particle size is similar to the effect of CH. Figures 1-3 show the TEM images of the plain liposomes. Morphologically, these plain liposomes were nearly spherical and had multilamellar structure characterized by multiple membrane bilayer. The candidate ratio for optimal liposome formulation was HPC : CH : Tween 80 (80:40:30, w/w), as a blank liposome. The mean particle size was ~ 382 nm and polydispersity index of 0.01, indicating a narrow size distribution and negatively charged at -28 mV. The

percentages of the HA entrapped into liposomes of 0.2 and 0.4 mg/ml of HA were as high as 75% and 79%, respectively.

CONCLUSION

Preparation of HA loaded elastic liposome by reversed phase evaporation has been achieved. The optimal liposome formulation was the ratio of HPC : CH : Tween 80 = 80:40:30, w/w. The mean particle size was 382 nm with negative charge of approximately -28 mV. Cholesterol is important to vesicle formation and can also act as membrane stabilizer (rigid membrane). The effects of CH and Tween 80 in increasing the particle size were similar which the mean particle size tended to reduce because the increasing CH and Tween 80 resulted in decreasing number of kinks in the bilayer and increasing the order of the lipid molecules (condensation effect) [6]. Entrapment efficiency of HA loaded elastic liposome for 0.2 and 0.4 mg/ml of HA was nearly as high as about 75-79%. In the future, the permeation of HA loaded elastic liposome will be carried out.

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