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Original Article

DEVELOPMENT OF CATHELICIDIN IN LIPOSOME CARRIER USING THIN LAYER HYDRATION METHOD

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ABSTRACT

Objective: The purpose of this study was to produce an optimum liposome formulation and to study the effect of formulation parameter such as phospholipid amount and hydration time on characteristics of liposome containing Cathelichidin.

Methods: Liposomes were prepared using a thin layer hydration method. Characterization of liposomes included organoleptic, PSA (Particle Size Analyzer) and zeta potential, entrapment efficiency, morphology by TEM (Transmission Electron Microscopy), the chemical interaction by FTIR (Fourier Transform Infrared Spectroscopy), and the stability by using Freeze-Thaw method.

Results: The result of the organoleptic test showed that the liposome were in the form of milky white dispersion, odorless, and without sedimentation. Optimum formula was obtained by making variations of soy oil: cholesterol 10: 0 (F1), 9: 1 (F2), 8: 2 (F3), 7: 3 (F4), and variations in sonication time (10 and 30 min). Based on the results, it was found that the optimum sonication time was 30 min. F2 and F3 were chosen as the most optimum formulas with particle sizes of 190.3±6.8 nm and 212.9±4.4 nm; polydispersity index of 0.192±0.023 and 0.137±0.022, and zeta potential as much as-38.8±0.6 mV and-34.8±2.0 mV. To the optimum formula, cathelicidin was loaded with hydration time varies of 100 and 120 min. Longer hydration time resulted in smaller particle size and higher entrapment efficiency either for F2 or F3. TEM characterization revealed a spherical shape of liposomes from the optimum formula. The results of FTIR characterization did not show any interaction between the phospholipids of liposomes with cathelicidin. The data from the stability test showed good stability for F2 and F3 with a hydration time of 120 min, indicated by a p-value>0.05, which indicated that there was no significant change in the zeta potential for three Freeze-Thaw cycles.

Conclusion: Formula of liposom using a variation of soy oil: cholesterol 9:1 and 8:2 with hydration time of 120 min revealed the best result with good stability for three Freeze-Thaw cycles.

Keywords: Cathelicidin, liposomes, Thin layer hydration

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INTRODUCTION

Cathelicidin is one of a group of Antimicrobial peptides (AMP) [1]. Cathelicidin is a small molecular peptide having a broad spectrum of antimicrobial activity and a function in the natural immune system as the first line of defense against microorganisms. In addition, cathelicidin has been shown to modulate wound healing by participating in angiogenesis, epithelial cell migration and proliferation, and immune responses [2, 3]. Antimicrobial peptides have received more attention due to their immense therapeutic potential. Although attractive for clinical applications, these agents have limitations in terms of stability and *in vivo* activity due to enzymatic breakdown by peptidases and interactions with anionic species of body fluids [4, 5]. AMP has low specificity so it is recommended to limit its therapeutic use to topical administration due to its hemolytic activity and toxicity at high concentrations [6]. In addition, most AMP are susceptible to chemical and proteolytic degradation under physiological conditions and require a protective matrix to allow efficient treatment [7–9].

Lipid-based drug delivery systems (LBDDS) have recently become very popular because of their remarkable ability to deliver drugs with poor absorption using lipids as carriers [10]. LBDDS are broadly classified as emulsion, vesicular system, and particulate lipid systems [11].

Liposomes offer unique possibilities to encapsulate and ability to protect peptides from degradation. Several studies have been conducted to overcome the limitations of cathelicidin [12]. Advanced nanotechnology has enabled nano-encapsulated structures as a strategy to minimize the undesirable characteristics of AMP [13]. It has been reported that peptides in nanoparticle structures exhibit lower cytotoxicity, reduced degradation and increased efficiency at the desired target [14, 15]. In a study conducted by Padmasree *et al.* (2022), Liposomes with PEG showed higher uptake by the tumor, but also toxicity was lower inside organs like the liver, kidneys, and spleen with PEG in mice with HT-29 colon carcinoma. Capecitabine stealth liposomes showed a prolonged circulation of drug in plasma, has increased the targeting of tumor and also improved therapeutic efficiency [16].

Other study conducted by Garcia-Orue *et al.* (2016), cathelicidin was encapsulated as Nanostructured Lipid Carrier (NLC) [2]. Rosenfeld *et al.* (2006) encapsulated cathelicidin peptides into Poly Lactic-co-Glycolic Acid (PLGA) nanoparticles [17]. Ron-Doitch (2016) conducted a study in which cathelicidin was encapsulated in a liposome carrier with surface modification [18].

Topical therapy is an excellent choice for the treatment of cutaneous infections due to its advantages, such as targeting of drugs to the site of infection and reduction of the risk of complete side effects [19]. Drug Delivery systems (DDS) have the ability to dissolve, entrap, encapsulate or to attach therapeutic agents into or onto their matrix, including small molecules, peptides, protein-based drugs, and nucleic acids, while their nanometric size allows them to overcome biological barriers and achieve cellular uptake [20]. In general, it is suggested that vesicle size ≥600 nm do not penetrate the deeper layers of the skin and stay in/or on the stratum corneum; vesicles ≤300 nm can penetrate more deeply, but vesicles ≤70 nm can deliver to both the viable epidermal and dermal layers [21].

Liposomes are the first generation of vesicular carriers that are non-toxic, biodegradable, biocompatible, and stable in colloid solutions in drug delivery systems. Cathelicidin encapsulation in liposome carriers is a promising alternative to optimize the administration of cathelicidin in terms of dose, delivery pattern, and safety. Liposome

system can protect the encapsulated peptides from protease degradation so that they can be administered via a topical route owing a risk of proteolytic breakdown by a number of bacterial proteinases [22].

MATERIALS AND METHODS

Materials

The materials used in the study consisted of cathelicidin (GL Biochem, Shanghai Ltd), soy lecithin (Archer Daniels Midlands), cholesterol (Dyeth), chloroform proanalysis (Merck), methanol, sodium phosphate monobasic monohydrate (NaH_2PO_4. H_2O) (BioWorld), and anhydrous dibasic sodium phosphate (Na_2HPO_4) (BioWorld).

Liposomes preparation

The liposomes were prepared using the thin layer hydration method. Soy lechitine and cholesterol were dissolved in chloroform: methanol (2:1) in a rotary evaporator at 40 °C at 60 rpm to form a thin film. The film was left overnight and then hydrated with phosphate buffer pH 7.4 at 60 °C at 200 rpm. Sonication times were 10 and 30 min (table 1).

Table 1: Liposome formulation

Formula	Soy lecithine (mg)	Cholesterol (mg)	Buffer (ml)
F1	100	-	20
F2	90	10	20
F3	80	20	20
F4	70	30	20

F1 = Soy: cholesterol (10:0), F2 = Soy: cholesterol (9:1), F3 = Soy: cholesterol (8:2), F4 = Soy: cholesterol (7:3)

To the optimum formula, cathelicidin was added (1.5% of the total lipid 2), using different hydration times (100 and 120 min). The relatively short hydration time resulted in larger size particles and less drug entrapment efficiency compared to longer hydration time [23].

Particle size reduction

Particle size reduction of liposomes was carried out by sonication using a sonicator probe. The sonication time were varied for each formula (10 and 30 min). Sonication were carried out at 0.5 cycles with an amplitude of 50% with treatment for 30 seconds on and 30 seconds off [24].

Characterization of liposome

Liposome were characterized by conducting the determination of Particle Size, Polydispersity Index, and Zeta Potential. Particle size and polydispersity index (PDI) were measured by (Dynamic Light Scattering) method using Zetasizer Zen3600 using aqueous media with 10 times sample dilution at room temperature [25]. Zeta potential (ZP) determination was employed to all formulations by Horiba SZ-100, Horiba Ltd., Japan.

Morphological observation

The morphology of liposomes was observed with negative transmission electron microscopy (TEM) staining. Briefly, a drop of the liposome suspension diluted with water (about 0.05 mg/ml) was placed on a 200-mesh Formvar copper lattice, allowed to adsorb and the excess removed by filter paper. A drop of 2% (w/v) uranyl acetate solution was added and left in contact with the sample for 5 min. Excess water was removed and the samples were dried at room temperature before the vesicles were imaged with a TEM operating at 200 KV [26].

Determination of encapsulation efficiency

Cathelicidin, which was entrapped in liposomes, were separated by ultracentrifugation (N-biotek-18000M small size high refrigerated centrifuge) at 15.000 rpm for 60 min. The supernatant were analyzed to calculate free cathelicidin using the Bradford test [27]. 40 µl of sample solution (centrifuged supernatant) was taken and placed in a 5 kDa all MWCO viva spin. After that, 2 µl of phosphate buffer solution pH 7.4 was added. 200 µl of Bradford's solution was added and homogenized. After five minutes, the absorbance were measured at 595 nm. The same procedure were conducted on the liposome blank and were measured in triplicate. Cathelicidinn were calculated using the formula [25]:

$$\%EE = \frac{\text{concentration of LL37 added} - \text{concentration of LL37 in supernatant}}{\text{concentration of LL37 added}} \times 100\%$$

Observation with fourier transform infrared spectroscopy (FTIR)

The interaction between cathelicidin and other chemicals in liposomes were evaluated by FTIR spectroscopy. Spectra were recorded using a Thermo Scientific Nicolet iS5 Infrared Spectrometer. The samples were poured on the FTIR plate and the spectrum were recorded at 4000 cm^{-1}–400 cm^{-1}. Then the spectra were compared to determine changes and interactions [25].

Stability testing

The liposome suspensions were stored at 4 °C for 24 h; then, the preparation were transferred at 40 °C for 24 h (1 cycle). The test were carried out in 3 cycles. Particle size, particle distribution and zeta potential were measured to determine the physical stability of the liposome suspension [28].

RESULTS AND DISCUSSION

In the manufacture of liposomes, a milky white dispersion of liposomes was obtained. The suspension were homogeneously dispersed and there was no precipitation at the bottom of the vial. The thin layer method was chosen in preparing the liposomes due to the ease and simplicity. Soy lecithin and cholesterol were used as the phospholipids to form the lipid bilayer in liposomes.

Cholesterol plays an important role in the composition of liposomes. Therefore, an optimization of the ratio of the amount of lipids (soy lecithin and cholesterol) had been carried out to obtain a physically stable formula that can be seen from the characterization results in the form of particle size, polydispersity index, and zeta potential values. Recent study proved that the addition of cholesterol improves physical stability of the liposomes.

The mechanism of the thin layer hydration method in liposome preparation consists of two stages, namely the formation of a thin layer and the hydration process. The thin layer were formed during evaporation in a rotary evaporator under vacuum conditions. This is adjusted for faster film formation due to faster evaporation of organic solvents. The temperature was set at 40 °C, which can provide the optimum temperature for evaporation despite the probability of thermal degradation. The formed film was hydrated using buffer phosphate pH 7.4. Hydration was carried out above the glass transition temperature of soy lecithin (50-60 °C) [29]. At a temperature of less than 50 °C, the soy lecithin dispersion forms a gel phase. While above the transition temperature will form a liquid crystal phase. The formation of liposomes occurs from the gel phase to the liquid crystal phase, which later will give each molecule the possibility to move more freely and then form a lipid layer into liposomes [29]. Determination of the ratio between soy lecithin and cholesterol (table 1) revealed that F1, F2, and F3 were easily hydrated and produced a visually good liposome dispersion. In contrast, the F4 was not perfectly hydrated and formed two-phase milky suspension. Based on visual observation, F2 and F3 are assumed as best formulas of liposomes. These results were supported by the results of particle size, particle size distribution and zeta potential characterizations.

Particle size and particle size distribution of liposomes

Particle size and particle size distribution of liposomes were measured using a Particle Size Analyzer (PSA) with the Dynamic Light Scattering (DLS) method. There are four formulas with variations in sonication time (table 1). Longer sonication time resulted in smaller particle size and polydispersity index (fig. 1). The optimum sonication time was 30 min with 30 seconds on and 30 seconds off.

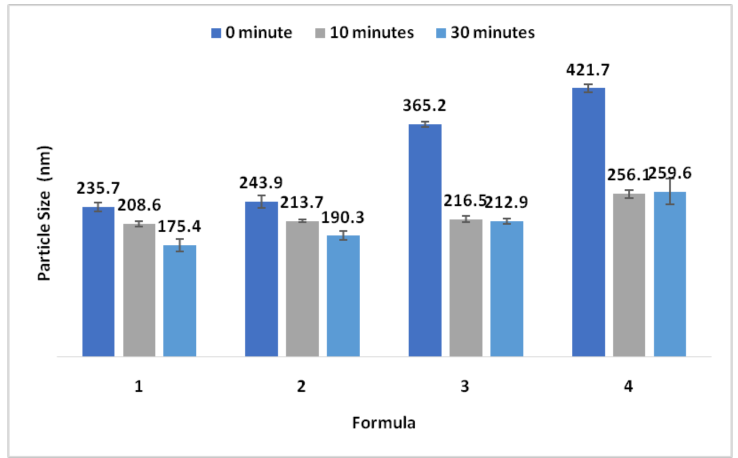

Fig. 2: Particle size of blank liposomes F1 soy: cholesterol (10:0); F2 soy: cholesterol (9:1); F3 soy: cholesterol (8:2), and F4 soy cholesterol (7:3) (results are expressed as a mean ±SD, n=3)

The results showed that an increase in hydration time resulted in smaller liposome-cathelicidin particle size. This result is in agreement with the principles that hydration is the phase of preparation to reduce the particle size of the liposome. Higher concentration of cholesterol used in formulation resulted in larger particle size, which due to more viscous emulsion during film formation as well as vesicle formation, which is caused by higher solid lipid content of cholesterol in the formulation.

Particle size distribution which is indicated by the Polydispersity Index (PDI) is a key aspect to be considered in the characterization of liposomes. The particle size distribution close to zero shows the good distribution of particles [30]. Small PDI values indicate the stability of the formula. Higher PDI value indicated that the particles are not uniform and tend to flocculate quickly. PDI value of<0.3 is considered to be acceptable and indicates a homogeneous population of phospholipid vesicles [31].

The values polydispersity index of the liposome samples can be seen in fig. 2.

The results of the polydispersity index of all samples after sonication for 30 min showed a homogeneous size distribution (PDI<0.3). Where F1, F2, F3, and F4 have PDI values of 0.177±0.049; 0.192±0.023; 0.137±0.022; and 0.212±0.048 respectively (fig. 2). In addition, the hydration time affects the amount of adsorption, to which the longer the hydration time, the greater the adsorption. Despite the increase in time of hydration, the polydispersity index was not affected. All formulas have a polydispersity index value that meets the requirements (<0.5, table 2).

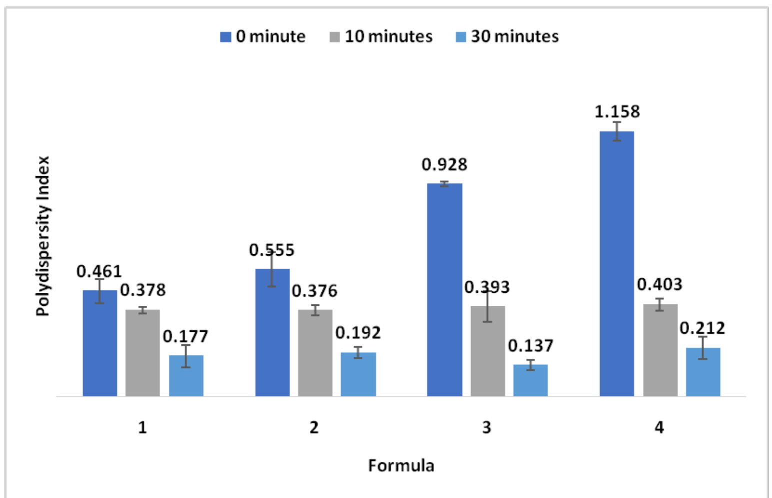

Fig. 2: Polydispersity index of blank liposomes with the formula F1 soy: cholesterol (10: 0); F2 soy: cholesterol (9: 1); F3 soy: cholesterol (8: 2), and F4 soy cholesterol (7: 3), (results are expressed as a mean ±SD, n=3)

Zeta potensial

Zeta potential is a parameter of electrical charge between colloidal particles. High value of zeta potential will prevent the particles from flocculation. Zeta potential value of±30 mV will provide good stability and a zeta potential value of±60 mV has excellent stability. A zeta potential value of about±20 mV only provides short-term stability, while a zeta potential value of±5 mV indicates rapid

aggregation [32]. All liposome formulations had negative zeta potential values (table 2). The zeta potential values of F1, F2, F3, and F4 were-25.6±3.5 mV, respectively; -38.8±0.6 mV; -34.8±2.0 mV; and-36.5±2.8 mV. Based on the results, it can be concluded that F2, F3, and F4 have good stability because they have zeta potential values of<-30.

Table 2: Results of characterization of blank liposomes*

Formula code	Particle size (nm)			PDI			Zeta (mV)
	Before sonication	Sonication 10'	Sonication 30'	Before sonication	Sonication 10'	Sonication 30'	
F1	235.7±6.6	208.6±4.0	175.4±9.5	0.461±0.053	0.378±0.014	0.177±0.049	-25.6±3.5
F2	243.9±10.1	213.7±2.2	190.3±6.8	0.555±0.073	0.376±0.021	0.192±0.023	-38.8±0.6
F3	365.2±4.0	216.5±4.9	212.9±4.4	0.928±0.010	0.393±0.066	0.137±0.022	-34.8±2.0
F4	421.7±6.4	256.1±5.7	259.6±20.7	1.158±0.041	0.403±0.027	0.212±0.048	-36.5±2.8

Results are expressed as a mean ±SD, n = 3

From the optimization results, the best formulas had been selected, namely F2 and F3. Next, these formulas were used for cathelicidin loading. Cathelicidin is hydrophilic; therefore, it was added at the time of hydration which was dissolved in phosphate buffer pH 7.4. The hydration time had been varied for F2 and F3. The Cathelicidin-loaded liposomes then were characterized by particle size, polydispersity index, FTIR, encapsulation efficiency, and accelerated stability test using *Freeze Thaw* method. The results of PSA characterization and zeta potential for cathelicidin-loaded liposomes can be seen in table 3, while that of entrapment efficiency are shown in table 4.

Table 3: Characterization of cathelicidin loaded liposomes*

Formula code	Particle size (nm)	PDI	Zeta (mV)
F2-LL37 (100')	239.8±5.9	0.286±0.010	-31.6±1.7
F2-LL37 (120')	228.5±5.6	0.234±0.035	-30.4±0.5
F3-LL37 (100')	261.1±6.9	0.311±0.053	-40.2±0.8
F3-LL37 (120')	251.9±6.1	0.329±0.013	-32.2±2.8

Results are expressed as a mean ±SD, n=3

Table 4: Encapsulation efficiency of cathelicidin loaded liposoom

Formula	%EE
F2a	67.0922±6.4561
F2b	87.1395±4.4022
F3a	79.7636±1.4869
F3b	97.3995±1.5335

F2a: F2-LL37 (100'), F2b: F2-LL37 (120'), F3a: F3-LL37 (100'), F3b: F3-LL37 (120'), results are expressed as a mean ±SD, n 3

The encapsulation efficiency analysis was aimed to determine the ability of liposomes to encapsulate cathelicidin at various concentrations of cholesterol. Based on the results, it was found that F3 produces a higher percentage of EE than F2. This can be explained by the higher content of cholesterol in F3 compared to that in F2. Cholesterol as hydrophobic solid lipid can strengthen the packaging of the phospholipid bilayer to become more rigid, thereby reducing membrane permeability.

FTIR analysis

FTIR analysis had been carried out to determine the functional groups in each material in the formulation. The FTIR spectra of soy lecithin, cholesterol, cathelicidin and liposomes were studied as shown in fig. 3. The spectrum of soy lecithin shown in table 5. These results are similar to the previously reported results [33]. The spectrum of cholesterol, liposome film, Cathelicidin, blank liposome as well as Cathelicidin-loaded liposome are respectively shown in table 6-9 and fig. 3.

Table 5: Spectrum of soya lecithin

Wave number range (cm^{-1}) [34]	Wave number based on spectra (cm^{-1})	Group
3000-2840	2922.72	C-H *stretching*
3000-2840	2852.23	C-H *stretching*
1750-1735	1741.40	C=O *stretching*
1465	1465	C-H *bending*
1140–1210	1166	PO_2 *groups stretching*

Table 6: Spectrum of cholesterol

Wave number range (cm^{-1}) [34]	Wave number based on spectra (cm^{-1})	Group
3550-3200	3392.78	OH *stretching*
3000-2840	2930.16	C-H *stretching*
1678-1668	1674	C=C *stretching*
1465	1463.33	C-H *bending*
1375	1376.12	C-H *bending*
	1054.53	*Ring deformation* [35].

Table 7: Spectrum of liposome film

Wave number range (cm^{-1}) [34]	Wave number based on spectra (cm^{-1})	Group
3550-3200	3291.82	OH *stretching*
3000-2840	3007.99	C-H *stretching*
3000-2840	2922.74	C-H *stretching*
3000-2840	2851.99	C-H *stretching*
1750-1735	1741.78	C=O *stretching*
1648-1638	1648.49	C=C *stretching*
1465	1462.28	C-H *bending*
1375	1376.23	C-H *bending*
1140–1210	1166.04	PO_2 *groups stretching*
	1052.23	Ring *deformation* [35].

Table 8: Spectrum of *Cathelicidin*

Wave number range (cm^{-1}) [36]	Wave number based on (cm^{-1})	Group
3310-3270	3284.83	Amide A (NH *stretching)*
3100-3030	2962.69	Amide B (NH *stretching)*
1700-1600	1647.88	Amide I (C=O *stretching)*
1580-1510	1540.04	Amide II (CN *stretching* dan NH *bending)*
1400–1200	1438.07	Amide III (CN *stretching* dan NH *bending)*
1205-1124	1135.47	C-O stretching

Table 9: Liposome blank spectrum and cathelicidin loaded liposomes

Wave number range (cm^{-1})[34]	Blank wave number (cm^{-1})	Sample wave number (cm^{-1})	Group
3000-2840	2923.17	2923.01	C-H *stretching*
3000-2840	2851.82	2852.01	C-H *stretching*
1750-1735	1741.00	1741.89	C=O *stretching*
1465	1463.16	1462.83	C-H *bending*
	1048.83	1052.69	*Ring deformation* [35]

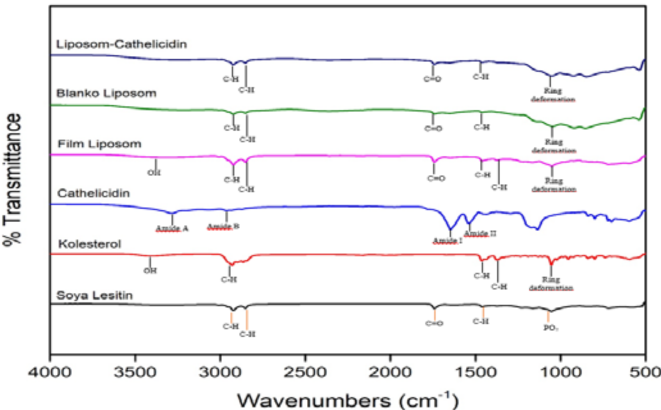

Fig. 3: FTIR spectra of (a) soy lecithin, (b) cholesterol, (c) cathelicidin, (d) liposome film, (e) liposome blank, (f) liposome-cathelicidin sample

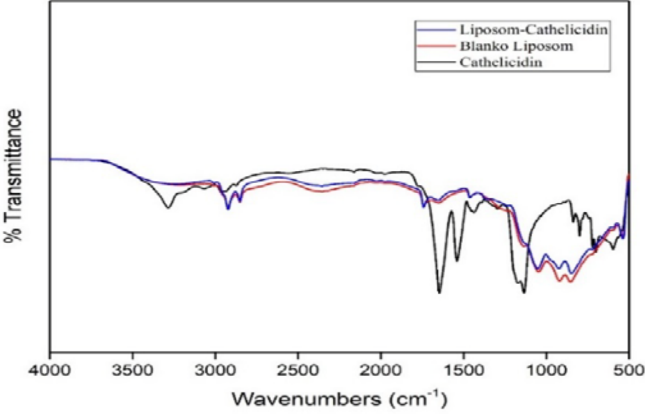

Fig. 4: Overlayed FTIR of blank liposomes, cathelicidin-loaded liposomes, and cathelicidin powder

The FTIR spectra of the blank liposomes and cathelicidin-loaded liposomes showed the same spectrum (fig. 3 and 4), which revealed that there was not any interaction between liposome-forming phospholipids with cathelicidin as the active substance.

Morphology study of liposomes

Morphological study of liposomes had been carried out using the Transmission Electron Microscopy (TEM). The samples used in this test were F2 and F3.

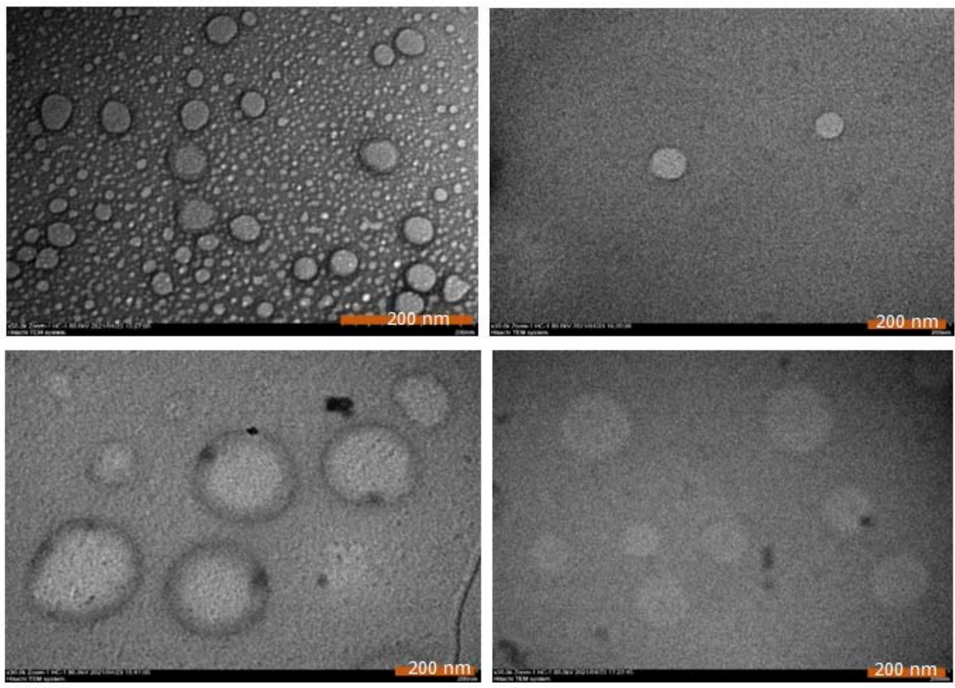

Fig. 5: Morphology of liposomes as (a) F2 with 50,000x magnification, (b) F2-LL37 with 30,000x magnification, (c). F3 at 30,000x magnification, (d) F3-LL37 at 30,000x magnification

Table 10: Results of freeze-thaw F2b and F3b

a. F2b

Cycle	Particle size (nm)	PDI	Zeta potensial (mV)
0	269.3±4.1	0.322±0.008	-34.8±1.9
1	270.7±8.0	0.367±0.040	-34.5±2.6
2	278.6±4.3	0.368±0.009	-31.2±2.4
3	291.1±6.2	0.376±0.004	-31.1±0.4

b. F3b

Cycle	Particle Size (nm)	PDI	Zeta Potensial (mV)
0	284.8±6.1	0.345±0.009	-36.7±2.0
1	298.2±9.0	0.354±0.009	-36.2±0.8
2	300.3±2.1	0.389±0.041	-35.0±1.6
3	311.1±5.9	0.407±0.009	-33.9±1.7

Results expressed as mean±SD, n=3

Fig. 5 showed that based on TEM determination, either blank liposome or cathelicidine-loaded liposome were spherical in shape. At a magnification of 50.000X, the liposome were individually dispersed.

Stability testing

Stability test were conducted using three cycles of Freeze-Thaw tests. The study were performed on F2, which produced with a hydration time of 120 min (F2b) and F3 with a hydration time of 120 min (F3b). Characterization on physicochemical parameter of the liposomes after each cycles of the Freeze-Thaw included particle size, polydispersity Index (PDI) and Zeta potential (table 10).

Statistical analysis were performed using IBM SPSS (Statistical Packages for Social Science) Statistics 23 software with Independent Sample T-Test. Based on the data, it was found that the particle size was significantly different, while the polydispersity index and zeta potential of F2b and F3b during cycle 0 to cycle 3 did not change significantly. The results of statistical analysis showed p value>0.05, which indicated that there was no significant change in the zeta potential and polydispersity index for 3 cycles of Freeze-Thaw. It can be concluded that cathelicidin-loaded liposome with the ratio of soy: cholesterol (9:1) and (8:2) with a sonication time of 30 min and a hydration time of 120 min had good physical stability because there was no significant change in the zeta potential value for 3 consecutive cycles.

CONCLUSION

The optimum formula of Cathelicidin-loaded liposome by using the thin layer hydration method was F2 and F3 with a sonication time of 30 min. Hydration time affected the physical parameters of

cathelicidin-loaded liposome, to which longer hydration time resulted in smaller the particle size and higher entrapment efficiency. Stability test which carried out by three cycles of Freeze Thaw method, revealed a good stability of liposomes in terms of the zeta potential values, which indicated that there was no significant change of liposomal electric charge after treatment of threr cycles of Freeze-Thaw.

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AUTHORS CONTRIBUTIONS

This work was carried out together among all authors. Author AYC and MKD carried out the experiments, analyzed the data and writing the manuscript. Author AYC Supervised the experimental design, Laboratory analysis for MKD and gave a major contribution in writing the manuscript. Author S contributes on formulation steps while author IMJ contribute in the production and characterization of liposomes. The contribution of RFD was as the funding provider due to her capacity as PI in the RISPRO Project. All authors read and approved the final manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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