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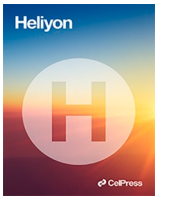

Preparation and molecular interaction of organic solvent-free piperine pro-liposome from soy lecithin

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ABSTRACT

Pro-liposome is a type of drug delivery system (DDS) with numerous advantages as a stable material with various applicability for several pharmaceutical dosage forms, to effectively deliver the material to reach its target in the human body. Nevertheless, it is mostly designed by employing an organic solvent hence giving rise to safety issues. We have developed a method for the preparation of organic solvent-free liposomes composed of soy lecithin and cholesterol by highlighting the importance of temperature during the initial mixing process, a self-hydration of a thin layer spread film, and a spray-drying technique with a suitable excipient as the carrier. The method was successfully applied to prepare a stable pro-liposome containing 0.17% (w/w) of piperine with an encapsulation efficiency of 95.58 ± 2.91%. Moreover, the study revealed that a piperine molecule forms hydrophobic interaction with six of the adjacent phospholipids in the liposome structure, this information can be useful for researchers designing similar studies. In conclusion, organic solvent-free pro-liposome can be an alternative method in the development of DDS, and several factors could be continuously improved to fulfill the intended pro-liposome characteristic.

1. Introduction

The application of a drug delivery system (DDS) in medication treatment become widely used due to its effectively delivering the active pharmaceutical ingredient (API) to desired target therapy in the human body. Drugs in DDS form are distinguished from their conventional system where the API is encapsulated in certain types of vesicles depending on its purpose, including prominent types such as liposomes, dendrimers, exosomes, solid-lipid nanoparticles, nanofibers, polymersomes, nanoemulsions, hydrogels, and others [1]. Among those types, liposomes have been successfully developed and approved by Food Drug Administration (FDA) to improve human health, including amphotericin B (Ambisome™, Abelcet™) injection, doxorubicin (Doxil™) injection, daunorubicin (DaunoXome ™) injection, lidocaine (ELA-Max ™) topical cream and continuously studied by researchers [2].

The artificial vesicles of liposome structure are composed of phospholipids and cholesterol, resembling the natural biological membrane. The phospholipid has a hydrophobic fatty acid tail and a hydrophilic phosphate head. Liposome formation begins in the presence of the water phase, leading to stronger intermolecular and intramolecular bonds of hydrophobic tails to avoid contact with water, thus forming a spherical form of the lipid bilayer liposome structure, with a polar phosphate group in the outer part of the liposome structure. The addition of cholesterol in the hydrophobic part of the structure is beneficial to regulate the properties of the

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bilayer membrane [3–5]. One of the advantages of liposomes is their ability to be designed to carry both water-soluble (hydrophilic drugs) and water-insoluble drugs (lipophilic drugs) by actively or passively loading without changing the active compound structure [2]. Nevertheless, most of the current conventional liposome preparation method has main disadvantages where it requires the use of an organic solvent such as chloroform, methanol, or ethanol during the dry layer formation of the lipid. Although solvent removal evaporation is employed during the lipid drying process, the risk of the residual solvent could remains. Such solvents are toxic to humans and should therefore be avoided. Due to that safety concern, several solvent-free liposomes had been continuously developed highlighting the selection of material and method including using advanced instrumentation. Several known techniques are high shear mixing dry powder lipids and dehydration-rehydration method, heating or Mozafari method, and pH jumping method [6].

Pro-liposome is dry form of liposome. It has liposome structure encapsulated in suitable carrier, mainly carbohydrate and dried by a certain method. A stable pro-liposome powder is acknowledge by its ability to form powder fulfilled the intended specification and able to immediately hydrated in contact with water or body fluid as dispersion system of liposome. Several method could be applied to form pro-liposome including slurry-based method, spray drying method, film deposition on carrier method, fluidized bed method, and supercritical anti-solvent method [7,8]. In smaller scale pro-liposome production, slurry-based method can be applied by dissolving the lipid in suitable organic solvent (i.e ethanol absolute, choloroform, methanol) and the active substance is incorporated into lipid phase, and the mixture is added into rotary evaporator flask containing carrier substance (i.e sucrose). Evaporation by using this instrument is conducted to remove the organic solvent and dried the liposome [9]. Meanwhile, in larger production scale spray drying method are preferable due to several advantages such as cost and time effective, also could prepare homogenous particle size in suitable controlled parameter. In this method, the suspension of liposome and carrier are atomized sprayed into drying chamber, and dried in single step [7]. The dry powder form of liposome will give advantages to enhance its stability compared to its liquid form, and also feasible to be applied in various pharmaceutical dosage forms including non-sterile and sterile preparation, as in solid, liquid, and semisolid dosage form [7,10,11].

Our current experiment employs soy lecithin and cholesterol to build liposome structure and piperine as the bioactive substance. Piperine is a bioactive alkaloid compound found in species of several families, including the leaves of *Rhododendron faurie* (Ericaceae), the dry rhizomes of *Zingiber officinale* (Zingiberaceae), and mostly found in the fruit of *Piper nigrum* L and *Piper albi* Linn (Piperaceae) [12,13]. The extracted piperine content varies from 2% to 9% in black pepper and 2%–7.4% in white pepper [12]. Piperine ($C_{17}H_{19}NO_3$) has a molecular weight of 285.34 g/mol. Around 97.5% of piperine occurs in the form of yellowish-white crystals with a weak pepper aroma and a pungent, spicy taste. Laboratory experiments confirmed that it has antimicrobial, antioxidant, immunomodulatory, anti-inflammatory, and anticancer activity [12,13]. Nevertheless, piperine shows low bioavailability and low biological activity due to its being poorly soluble in water (0.004 mg/ml at 18 °C) [14,15]. Therefore, various optimization methods have been developed to improve its solubility and bioavailability, by synthesizing the piperine derivative, complex formation, co-crystallization, particle size reduction, and encapsulation with lipid- and polymer-based drug delivery systems [16].

In this context, this study investigated the optimization of the preparation of piperine pro-liposomes without the use of organic solvents via a simple process thus feasible for up-scaled to an industrial scale.

2. Materials and methods

2.1. Materials

Piperine with >97% purity and cholesterol were purchased from Sigma-Aldrich (Saint Louis, USA), and soy lecithin was purchased from Solae (Solae, Brazil). Tween 80 (Polysorbate 80) was purchased from PT Brataco (Indonesia), Maltodextrin DE 10–12 was purchased from Qinhuangdao Lihua Starch Co.Ltd (China) and sucrose from Merck (Germany).

2.2. Preparation of piperine liposomes

Liposomes were prepared by thin layer film hydration technique, similar to the thin-film hydration method but applied to the undried lipid. The lipid phase components, including soy lecithin and cholesterol, were accurately weighed at a ratio of 12:1 and mixed at 60 ± 5 °C. After the cholesterol was completely dissolved, 300 mg of piperine was added, followed by mixing at 60 ± 5 °C. The lipid phase was hydrated by pouring it to form a thin layer above the water phase containing 2% of tween 80 at 25 ± 5 °C and allowed the mixture to hydrate overnight. Following this, the nano-to-micrometer size of liposomes can be achieved by stirring with a magnetic stirrer at a low speed.

Table 1
Formulation of pro-liposome with different carrier agents.

Raw material	F1 (gram)	F2 (gram)	F3 (gram)
Soy lecithin	12	12	12
Cholesterol	1	1	1
Piperine	0.3	0.3	0.3
Sucrose	72	32.5	43.3
Maltodextrin	–	32.5	21.7

2.3. Effect of carrier agent protection of piperine pro-liposomes and rehydration evaluation

The liposome solution was subjected to further processes by a spray-dried method using a BÜCHI Mini Spray Dryer B-290 with an inlet temperature of 123 °C, an outlet temperature of 61 °C, an aspirator at 90%, and a pump at 25% with the aid of carrier agent. Several combinations of carrier agents were tried as seen in Table 1. F1 was designed with a weight ratio of lipid: carrier 1:6, meanwhile carrier agent in F2 and F3 was slightly reduced to 1:5.4 with a combination of sucrose and maltodextrin to avoid the sticky concern of the sucrose and increase the yield.

The morphology of the piperine liposome before the drying process and rehydration of the pro-liposome were analyzed using an inverted phase contrast microscope (Olympus, Japan) at 400x magnification. The rehydration of pro-liposome was conducted by dispersing 250 mg of pro-liposome in 250 μl of deionized water and gently shaking to completely disperse.

2.4. The production yield of piperine pro-liposomes

The percentage yield of the pro-liposomes calculated using the following equation:

$$\boldsymbol{PY}(\%) = \left(\frac{\mathbf{Wp}}{\mathbf{Wt}}\right) \text{ x } 100$$

where PY is the production yield, Wp is the piperine pro-liposome powder, and Wt is the total theoretical weight of materials present in the formula excluding water, including the total weight of sucrose, piperine, soy lecithin, cholesterol, and tween 80.

2.5. Analysis of piperine pro-liposome via scanning electron microscopy (SEM)

The morphology of the piperine pro-liposome powder obtained from formula F1 was examined using a scanning electron microscope (SEM) (TM3000 Tabletop microscope HITACHI, Japan). The pro-liposome was placed onto carbon tape and positioned on the stub. The sample stub was inserted into the chamber and operated at a low vacuum of 15.0 kV and an emission current of 47,800 nA. The filament current of 1,750 mA magnification was maintained and collected for analysis and observation at magnifications of 300× and 2,500 × by the 02–03 software version.

2.6. Characterization of the particle size, charge, and zeta potential of the piperine liposome solution

The size distribution and zeta potential of the liposomal formulations (formula F1) were characterized by dynamic light scattering (DLS), using a Nano Particle analyzer (SZ-100 Nano Partica, Horiba Scientific). The measurement was performed through a helium-neon (He–Ne) laser beam with a scattering angle of 90° and a holder temperature of 25 °C. The samples were diluted with deionized water to reach the phospholipid concentration of 3,000 ppm. The particle size, liposome charge, and zeta potential were analyzed in triplicate, and the data were analyzed using the HORIBA SZ-100 with dynamic light scattering technique by Windows [Z Type] v. 2.40 software.

2.7. The entrapment efficiency of liposome piperine solution

The entrapment efficiency of piperine in liposomes was analyzed in formula F1. One ml of samples (before the addition of sucrose) was taken from the middle part of the solution and transferred to a 10-ml volumetric flask. Subsequently, add methanol to reach a volume of 10 ml. The piperine standard was prepared in methanol at a concentration of 5 ppm. Samples and standard solutions were filtered through a 0.45-μm PTFE membrane filter (Advanced Microdevices). The solutions were analyzed using the Waters Alliance HPLC system with a Photo Diode Array (PDA) detector, and the results were processed by the Empower® 3 software. Chromatographic separations were done on Jupiter®, C-18 (5 μm particle size, 250 × 10 mm i.d.), with a 20 μl volume injection. The mobile phase consisted of acetonitrile-methanol-water (65:5:30) at a flow rate of 1.0 ml/min. The chromatogram was recorded at 342 nm [17], and the entrapment efficiency (EE %) was calculated using the following formula:

$$EE\% = \frac{amount\ of\ piperine\ in\ 1\ ml\ liposome}{amount\ of\ teoritical\ piperine\ in\ 1\ ml\ liposome\ formula} \text{ x } 100\%$$

2.8. Assay of piperine in pro-liposome

First, 100 mg of pro-liposome (formula F1) was weighed and transferred to a 10-ml volumetric flask. Subsequently, 3 ml of water was added, followed by shaking. Methanol was added up to 10 ml. The standard solution, mobile phase, and chromatographic system were conducted according to the method of Entrapment efficiency.

2.9. Molecular docking complex of piperine in phospholipid bilayer liposome

The mechanism of piperine binding in liposomes was studied by molecular docking using AutoDock 4.2 software. The crystal structure of the lipid bilayer of *Escherichia coli* K12 [PDB ID: 6CSX] refers to PDB (https://www.rcsb.org/structure/6CSX) and was

employed as a representative of the phospholipid bilayer liposome. This lipid bilayer structure is composed of several types of phospholipids including phosphatidylethanolamine which facilitates our study of its similarity with phospholipids of soy lecithin. The three-dimensional structure of piperine was obtained from the PubChem database (CID: 638024). The ligand has three active torsions and the center of the coordinate grid map was arranged at x = −137.628 y = 143.207 and z = 108.256) with a 58 × 64 × 66 point grid map. The Lamarckian genetic algorithm was selected and 25 docking runs were performed, with 150 population size and medium 2.5 × 10^6 evaluation energy. Molecular docking was continued using the lowest conformation binding energy.

3. Result

3.1. Preparation of piperine liposomes

Of several methods tried in our laboratory (unpublished report), nevertheless, thin film self-hydration of the lipid phase with water is preferable to hydrating the lipid mixture in this formulation. The thin lipid phase of piperine liposomes was found will be immediately hydrated once exposed to the water phase indicated by a change of a dark brown layer into beige (Fig. 1A). The thinness of the film will shortly form liposomes than a thick film due to the increment of surface contact with water. The liposome solution obtained after particle reduction and homogenization by stirring was characterized by a milk-like beige-to-yellowish liposome solution with a pH of 6.4 (Fig. 1B). At this stage, there was no piperine crystal observed in the water phase observe visually, and the aid of a microscope indicates self hydration method was preferable to maintain the entrapment of piperine in the hydrophobic region of the multilayer liposomes.

3.2. Effect of carrier agent protection of piperine pro-liposomes and rehydration evaluation

The liposome solution was successfully dried into powder by spray-dried technique, resulting in dry powder. Nevertheless, among several carrier agents applied, only F1 containing sucrose successfully form a good pro-liposome characteristic with no leakage nor breaking of structure. Therefore, further study was employed on the F1 pro-liposome. pro-liposome obtained from F1 was

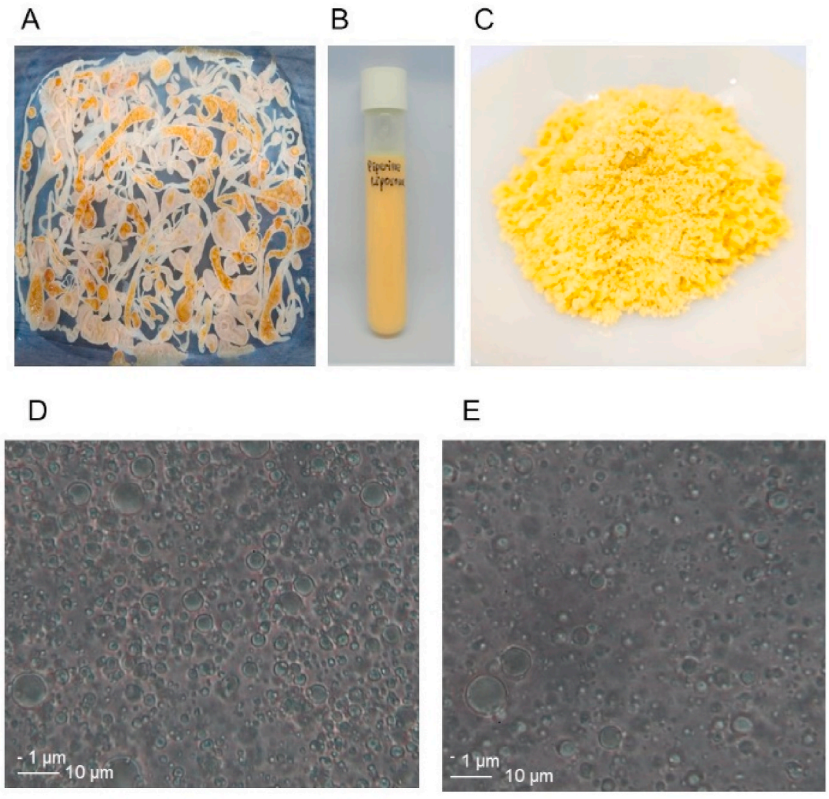

Fig. 1. Preparation of piperine liposome by thin layer film hydration and microscopic evaluation of liposome and rehydration of pro-liposome. Preparation of piperine liposome by thin layer spread film hydration allows self-hydrated after approximately 2 h depending on the thickness of the film (A). The hydrated film was stirred to prepare milk-like beige-to-yellow liposome solution (B) and further process as pro-liposome by spray dried method (C). Piperine Liposomes and rehydyration of pro-liposome were successfully prepared in nano to micrometer size as spheric shape (D and E).

characterized as a light yellow powder, the sweet and biting taste of pepper, and a very weak specific pepper odor, with a limitation of hygroscopic characteristics due to sucrose contained in the powder (Fig. 1C).

The liposomes were successfully generated in a spherical shape and in the size of nano to micrometers even without organic solvent (Fig. 1D). The pro-liposomes successfully formed the liposome with a size similar to that before the drying process (Fig. 1E).

Sucrose exhibited an excellent protective effect on liposomes during spray drying by microscopic analysis (Fig. 2A). Meanwhile, microscopic observation showed there was leakage of liposome structure in F2 and F3 (Fig. 2B). The presence of maltodextrin improved the sticky problem during the spray dry process and the flowability of the powder, thus affect to yield improvement. Nevertheless, its protection effect required further study. This study had been indicated that the optimum weight ratio of lipid: sucrose to protect liposomes designed as in Table 1 is 1:6. F2 and F3 have a combination of sucrose: maltodextrin 1:1 and 2:1 thus weight the ratio of lipid: sucrose 1:2.7 and 1:3.6, respectively. Leakage of liposome structure as seen in F2 and F3 might be due to lack of sucrose and the lower protection effect of maltodextrin on liposome structure due to steric hindrance effect (Fig. 2B).

3.3. The production yield of piperine pro-liposomes

The theoretical solid material in F1 was 91.3 g, nevertheless, the powder mass obtained from the drying process was 30.7 g or 33.6% with most of the pro-liposome adherent to the wall of the drying chamber. The presence of maltodextrin in F2 and F3 significantly improve the yield to 52.4% and 44.8% in F2 and F3, respectively.

3.4. Analysis of the piperine pro-liposome via scanning electron microscopy (SEM)

Scanning electron microscopy observation of the F1 piperine pro-liposome showed aggregation of sucrose in an irregular shape (Fig. 3A). The individual small smooth surface of sucrose covering liposome was identified (Fig. 3B). Nevertheless, most of the topological structure of the powder suggested that the small liposome was covered with sucrose and agglomerated into clump powder. There was no needle shape of the piperine crystal observed, indicating that there was no leakage in liposome structure during the process and the piperine remained in the liposome during the process.

3.5. Characterization of the particle size, charge, and zeta potential of the piperine liposome solution

The mean size of the piperine liposome obtained was 199.8 nm, with a polydispersion index (PI) of 0.594 (Fig. 4A) and zeta potential value of −13.3 mV (Fig. 4B). A PI value was less than 0.7 indicate that the samples prepared with a phospholipid concentration 3,000 ppm were suitable to be analyzed by this dynamic light scattering method.

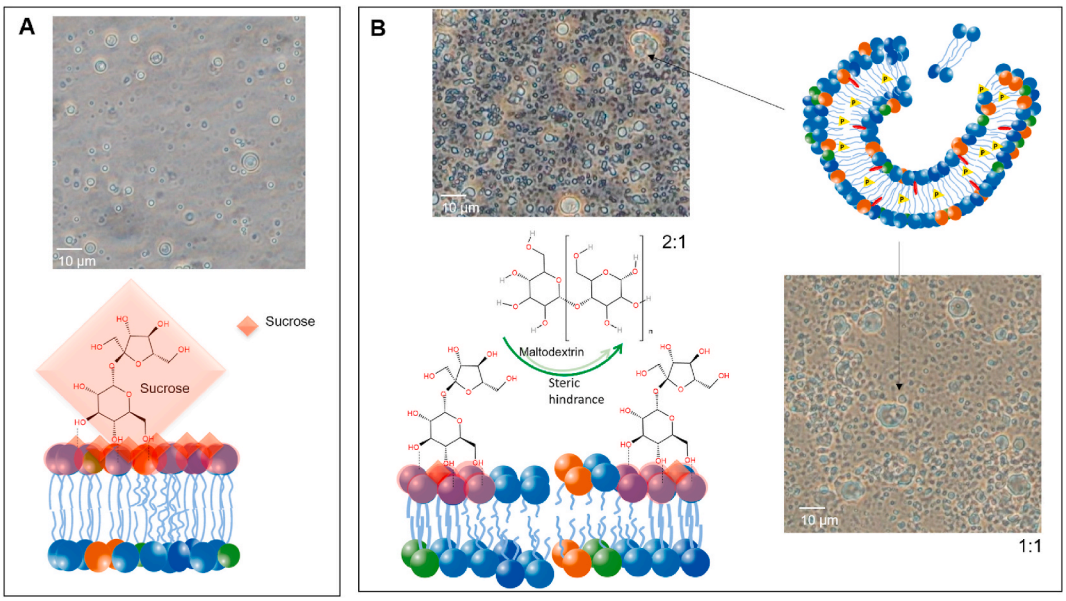

Fig. 2. Mechanism of carrier agent protecting liposome structure during the spray drying process. Sucrose protects liposomes during process by forming a hydrogen bond with the head of phospholipid groups on the surface of liposomes, and fill the gap of phospholipids structure occurred at high temperatures due to a decrease of intermolecular bonding (A). Maltodextrin has less ability to form a hydrogen bond with polar head due to steric hindrance phenomenon, thus the combination of Sucrose: Maltodextrin 2:1 and 1:1 reduce its protective effect and cause breaking of phospholipids membrane (B).

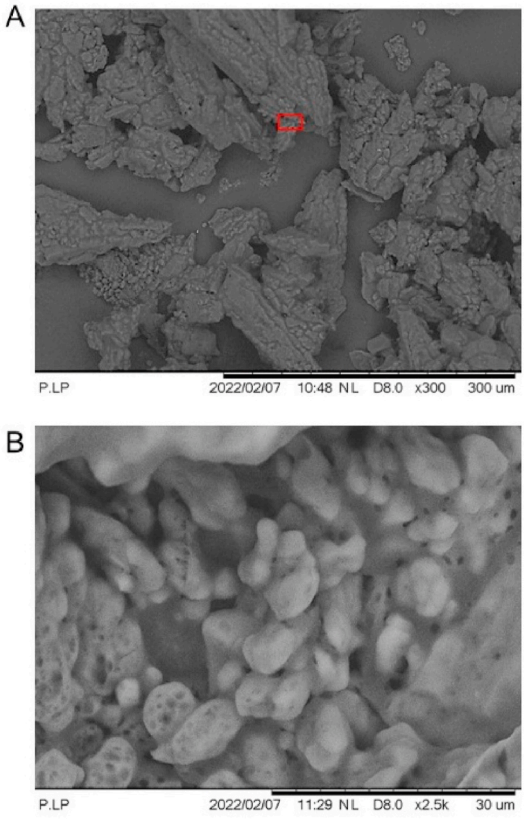

Fig. 3. The morphology of piperine liposome powder under a Scanning electron microscope (SEM) at 300x magnification shows small liposome agglomerated into clump powder (A), and at higher 2500x magnification shows the topological structure of liposome powder covered by sucrose (B).

3.6. The entrapment efficiency of liposome piperine solution

HPLC analysis result showed the piperine contained in liposome solution (without sucrose) 0.95 ± 0.02 mg/ml of the 1.0 mg/ml loaded piperine, resulting in an entrapment efficiency of 95.58 ± 2.91%. The chromatogram of piperine with a purity of 97.5% at 5 ppm resulted in the main peak at the retention time of 5.796 min (Fig. 5A). The piperine liposome solution analyzed showed a similar profile, with the main peak at a retention time of 5.715 min; small peaks appeared before and after the main peak was derived from the

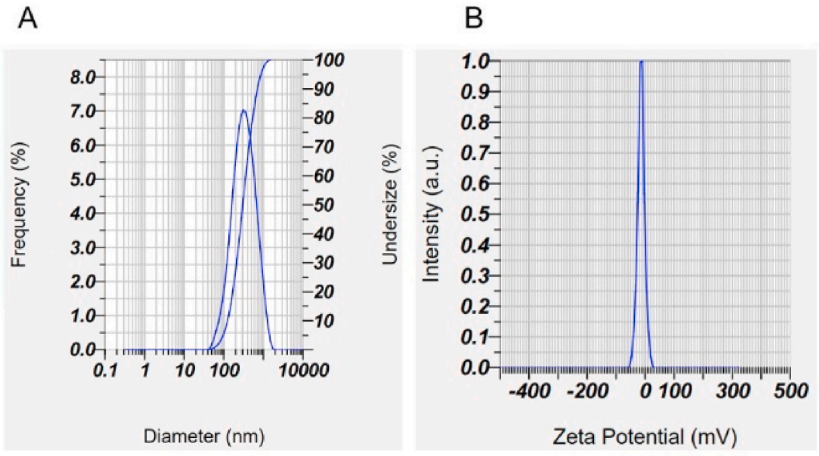

Fig. 4. The size distribution of piperine liposome at lipid concentration 3000 ppm analyzed by using a particle size analyzer shows that the mean size 199.8 μm with Polydispersion index (PI) = 0.594 (A) and zeta potential value of the liposomal is −13.3 mV (B).

excipient used (Fig. 5B).

3.7. Assay of piperine in pro-liposome

HPLC analysis of pro-liposome assay piperine content in powder as 0.17 mg in 100 mg of powder or 0.2% (w/w) with the main peak of piperine appearing at 5.729 min. The theoretical assay was 0.3% (w/w) (Fig. 5C).

3.8. Molecular docking complex of piperine in phospholipid bilayer liposome

The macromolecule employed in this study comprises less phospholipid in the upper layer than in the inner layer (Fig. 6A). Interestingly the study showed a piperine preference for binding to the inner lipid bilayer with extra phospholipids, which allows it to form a hydrophobic bond with several phospholipid molecules. One molecule of piperine was found to create hydrophobic bonding with six molecules of phospholipids (Fig. 6B). The amide moiety of piperine structure creates alkyl interaction with adjacent molecules, specifically with 2 molecules of PE (green and light green color) at C14 and C36 in 4.6 Å and 4.67 Å distance, and with PA molecules (cyan color) at C16 3.97 Å. Moreover, benzene of aromatic moiety of piperine forms Pi-alkyl interaction with four adjacent of PE molecules. Interaction with 13 and C16 at 4.94 Å and 4.92 Å of yellow and orange PE molecules, and with C32 and C35 at 3.88 Å and 5.09 Å of pink and green PE molecules. Conventional hydrogen bond interaction occurs to link the head of PE (indicate with green and light green color) at 2.81 Å. Hydrophobic bond interaction also occurs between fatty acid chains at distances 4.33 to 4.76 Å indicating a strong intermolecular bond of phospholipids pack. Of the 25 conformations, conformation 9 has the lowest energy As shown in Fig. 7, the structure of piperine allows it to form a complex with a lipid bilayer with ΔG – 6.44 kcal/mol and inhibition constant Ki of 18.98 μM.

4. Discussion

Piperine is very slightly soluble in water (0.04 mg/ml at 18 °C) and highly lipophilic in nature, resulting in a low dissolution rate

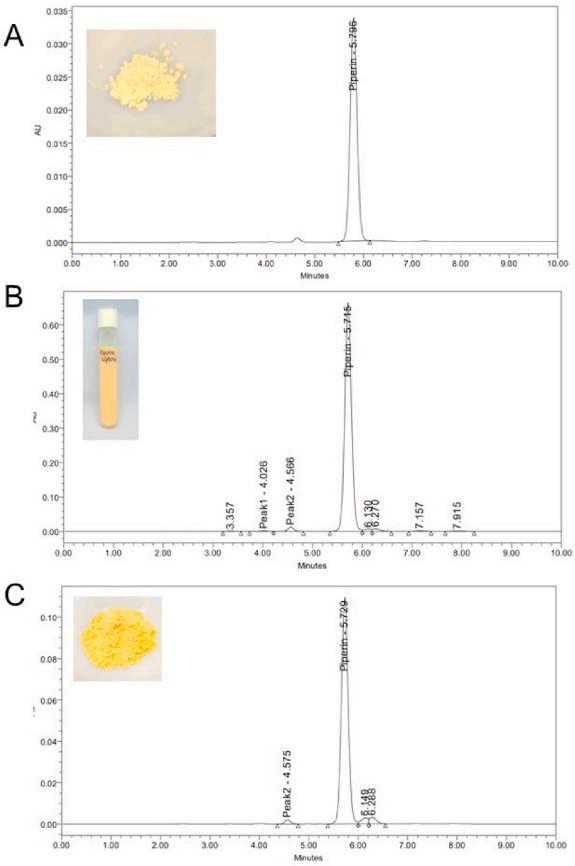

Fig. 5. The Chromatogram of piperine analyzed by HPLC shows the main peak at a retention time of 5.796 min (A). the chromatogram was used to ana the encapsulation efficiency of piperine liposome (B) and liposome piperine powder (C).

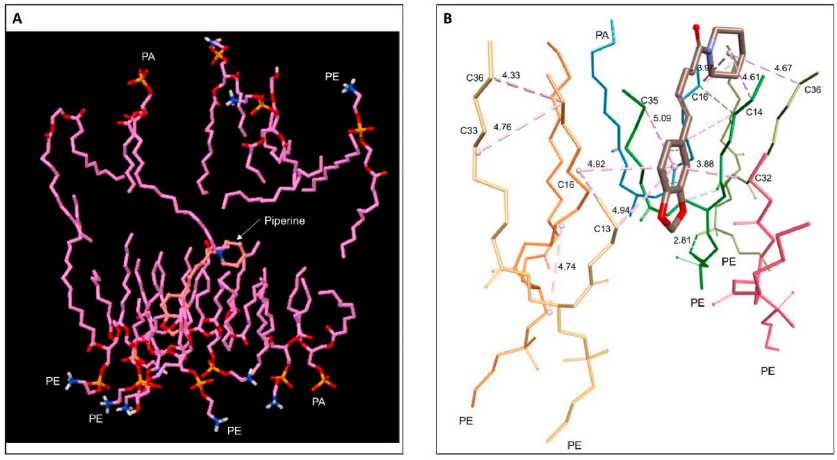

Fig. 6. Piperine binding and interaction in phospholipids of liposome structure. Interestingly the study showed a piperine bind to the inner lipid bilayer (A) and form interaction with several phospholipid molecules (B).

and low bioavailability in the body [14]. Hence, encapsulation of piperine in a liposome as a drug delivery system is expected to facilitate its transport across the natural lipid bilayer membrane and improve its bioavailability in the human body. Liposomes are also known as one of the most successfully developed DDS approved by FDA as medicine for several treatments [2]. Depending on the characteristic of lipid in the formula, most of liposome is prepared by employing a certain organic solvent to facilitate dissolve and interaction of lipid with active substance during liposome formation. Ethanol is an example of solvent usually applied in liposome preparation composed of synthetic lipid such as 1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine (DOPE), L-a-phosphatidylcholine, hydrogenated soybean phospholipids (HSPC), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine. Moreover, in order to reduce it to nanoparticle size, several types of equipment is required including a dynamic microfluidic machine, high shear homogenizer, and sonicator [16,18,19].

There are several types of lipids including synthetic and natural lipids as an alternative to construct the liposome structure. Soy lecithin is one of the natural lipid commonly used in the study of DDS, also as an alternative to egg- and bovine-derived lecithin due to its lower cost, higher availability and lower microbial risk, lower the risk of pathogenic and virus contamination [20]. Different from pure synthetic phospholipid, soy lecithin is composed of several phospholipids as 29–46% of Phosphatidylcholine (PE), 21–34%

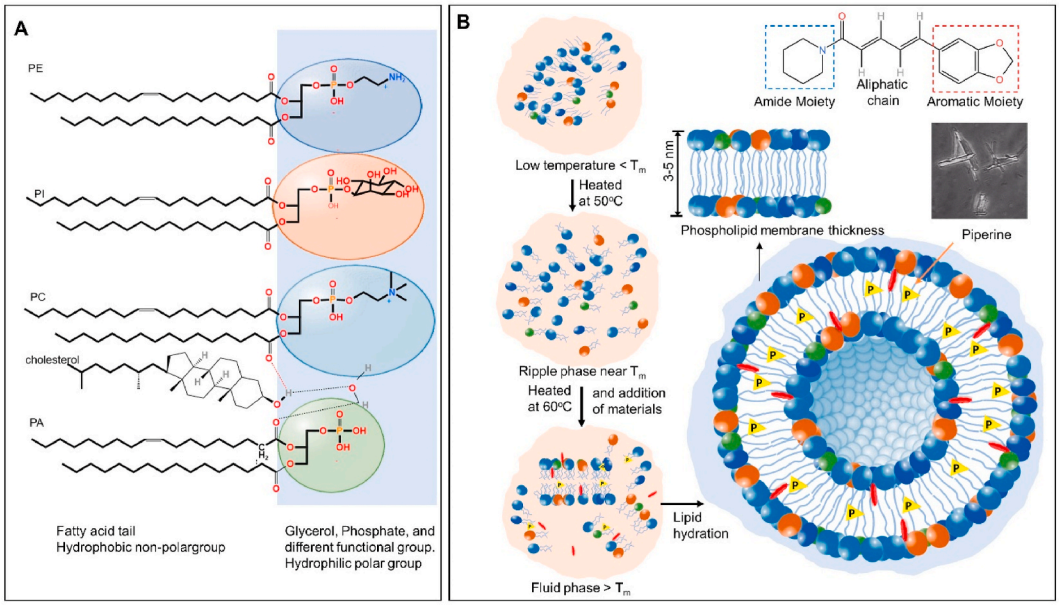

Fig. 7. Phospholipids of soy lecithin and cholesterol-composed liposome piperine structure. The main phospholipids of soy lecithin known as Phosphatidylethanolamine (PE), Phosphatidylinositol (PI), Phosphatidylcholine (PC), and Phosphatidic acid (PA), have similarities in the hydrophobic fatty acid tail and polar structure containing glycerol, and phosphate, with distinct functional groups attached to the phosphate group (A). Suitable temperature is required for mixing the material and form liposome structure (B).

Phosphatidylethanolamine (PE), 13–21% Phosphatidylinositol (PI), approximately 8% of Phosphatidic acid (PA) [21–23]. Phospholipids are esters of glycerol, they have similar fatty acid chains as hydrophobic tails and hydrophilic polar heads of glycerol, phosphate, and alcohol. Different types of amphoteric phospholipids are distinguished by types of functional groups attached to phosphate groups, and affecting the expression of head group charge as negative or basic (Fig. 7A). Moreover, the charge is also affected by the pH of the environmental liquid., for example, PC has a neutral charge molecule and zwitterionic at physiological pH. Similarly PC and PE is also zwitterionic at physiological pH, however, it turns into a negative charge at pH basic [23–25]. Our experiment revealed that 40 mg/ml of lipid in a solution containing 2% Tween (a nonionic surfactant) has pH 6.4 and a negative charge of −13.3 mV. Designing liposomes whether in neutral, positive, or negative charge also important to achieve maximum permeability of the drug delivered to its target. The positive charge liposome able to reduce the possibility of phagocytosis by macrophages [26]. Meanwhile, the negatively charged liposome is preferably designed for topical preparation to allow immediately diffused permeation to the skin into the dermis after its application which is reported faster than positively charge liposome penetration [26]. Another example is gentamycin liposome, it is designed as neutral liposomes to enhance their antimicrobial activity against planktonic bacteria, and as negatively charged to increase encapsulation and activity against the biofilm community [27]. Considering the piperine pro-liposome resulted has negative charge, thus it application in topical dosage form could be further studied.

In the absence of an organic solvent or polyol to dissolve the lipid mixture, applying a suitable temperature to the lipids mixture and other compounds is a critical. We revealed that mixing at temperature of 60 ± 5 °C was effective to combine soy lecithin and cholesterol in the ratio of 12:1 (w/w), for a stable vesicle liposome with high encapsulating efficiency. The liposome formation process in our experiment is described as a schematic diagram as seen in Fig. 7B. Several studies report various Transition temperatures (T_m) of Soy lecithin from 50 to 65 °C, which is affected by the length of fatty acid phospholipid and purity of material [28,29]. At low temperatures below Tm, the soy lecithin phospholipids are in non-bilayer structure and insoluble in water. Heating the soy lecithin near T_m allow heat absorption and give sufficient energy against intermolecular force. This energy loosen up the hydrophobic bond, and ripple the fatty acid tail due to presence of double bond in the structure. Further heating of the phospholipids at 60±5 °C (T_m 50–65 °C) affects the higher energy absorption and thus increasing the fluidity of the soy lecithin's phospholipids. This phase is known as the fluid phase and is important to allow the interaction of cholesterol and piperine with the fatty acid chain [3,30–32]. Subsequently, fluid phase of was poured above the water phase as a thin film. Preparation of thin film of lipid layer is important to allow spontaneous formation of liposome structure, faster hydration and smaller size of liposome particles [33]. Nevertheless, the limitation of this experiment was due to unavailable equipment to prepare uniform thickness of the film. Thus overnight hydration was expected to achieve optimum hydration of thickest film. Applying magnetic stirrer at 300 rpm to allow consistant agitation was successfully reduces the particle size of liposom and maintain the piperine entrappement in liposome structure. The presence of cholesterol known has an important function to support the flexibility of liposomes by creating hydrogen bonds with water solvents and direct hydrogen bonds with a fatty acid tail (Fig. 7A and B) [34].

Liposome is designed to mimic the extracellular vesicle (EV), and due to their similarity, the liposome may transfer the drug to EV and transport it to the designated organ. The EVs are known to have particle sizes 40–100 nm, thus the liposome with sizes 10 nm–250 nm and most preferably 40–100 nm is suitable and widely applied as a sterile injectable dosage form for cancer treatment. Meanwhile, the larger size of 1–5 μm is optimum size for oral administration pulmonary drugs [35–38]. In general, the thickness of the single layer of phospholipid bilayer is approximately 3–5 nm, thus engineering the liposome in 25 nm is possible and known as small unilamellar vesicles (SUVs). Albeit, liposome is generally design in nanometer size, several liposome with bigger sizer (e.g 31 μm of multivesicular liposomes (MLVs)) is also approved by FDA for specific treatment [5,34,37,37,39,40].

The liposome obtained in this experiment was vary from nano to micrometer size, could be developed further to obtain the suitable intended size. In our experiment, lipid concentration prepared in concentration of 40 mg/ml, thus resulted dense liposome solution as seen in Fig. 1B. Higher concentration will be effective during spray dry process to remove water. Nevertheless, large unilamellar vesicles (LUV) to MUV liposome was observed. This limit its application only to nonsterile liquid, semisolid, or solid preparation, as a topical or oral dosage form. Our laboratory study informed that preparation of a thinner layer will be beneficial to increase surface contact and allow faster and optimum hydration for smaller SUV size of liposome particles. A study in agitation speed and time would also be useful, however further study must be conducted due to the risk of liposome structure breaking and leading to piperin out of liposome structure. Moreover, since lipid concentration significantly will affect the resulting liposome particle size, a study in more diluted the lipid concentration to 0.5–2.5% would also be useful for this purpose [41,42]. Related to this matter, where lowering the concentration and increase frequency of agitation may reduce the particle size of liposome, this phenomenon also affecting result of particle size measurement conducted by particle size analyzer. Since preparation of sample for those analysis required dilution of lipid from initial suspension into 3,000 ppm, and gentle agitation for sample homogenization. Thus the analysis result particle size 199.8 nm, smaller than optical microscopy observation. This is also indicate that further research to resize into smaller size of liposome is possible.

Our experiment employed spray-drying method for pro-liposome preparation, considering the stability of formula to heat, time-efficient, and less expensive. The biggest challenge in this technique was maintaining the integrity of the liposome structure against harsh conditions of high temperature, vacuum, and particle movement during the process. Therefore the selection of suitable types of carrier agents, its quantity (lipid to carrier ratio) and the temperature of the inlet-outlet is important to avoid the leakage of the structure. Maltodextrin, trehalose, sucrose, lactose, and mannitol are known as alternative of carrier in spray dry technique [43,44]. Nevertheless, the compatibility of these carriers to each formula could show a different result. Compare to maltodextrin, the inclusion of sucrose in our formula with the ratio of lipid sucrose (1:6) showed a better result, indicated by a result of rehydration test where the hydrated powder showed liposome in the original spherical shape, free of piperine crystals and debris (Fig. 1E). The result relevant with the previous study that small size of sucrose molecule with a molecular weight of 342.30 g/mol and glass transition temperature

(Tg) of 60 °C, allow to reach and interact with phospholipid head group via hydrogen bonding and thus has the flexibility to protect the liposome under stress drying conditions at 123 °C by the formation of sugar glass on the liposome surface (Fig. 2A) [43,45–47]. Meanwhile, the addition of maltodextrin as a nonadsorbing polymer with Tg higher than that of sucrose at 162 °C may prevent the powder from becoming sticky during the drying process and better yield [47,48]. Nevertheless, the larger size of DE 10–13 maltodextrin which has a larger size of 1800 g/mol, has steric hindrance of thus unable to reach the phosphate group and give protection during the drying process [49–52]. In addition, several studies also reported less protection from maltodextrin applied in a concentration of 20% (b/v). Negatively charged of maltodextrin as hydrophilic nonionic polymer also known could potentially alter the liposome structures, flocculation, break down, and slightly reduced zeta-potential values [44,53,54]. Moreover, to obtained better yield and avoid the sticky of pro-liposome in the drying chamber, the concentration of sucrose was reduced and substituted with maltodextrin. The yield was improved, however broken the liposome structure was observed. Therefore, we postulate that maltodextrin fail to form a viscoelastic film between and on the surface of the head phospholipid, thus allowing the heat exposure to reach the unprotected liposome structure during heat exposure to at123 °C and result in the liposome leakage [55]. Further study would be necessary to combine suitable carrier agents to obtain higher yields while maintaining liposome functional structure.

Docking analysis employs the lipid bilayer of escherichia coli K-12 available from the crystalization experiment (PDB 6CSX). It comprises 19 lipid molecules in a bilayer structure with 7 irregular ordered packing of lipids in the outer leaflet, and 12 hexagonal arrays of inner packed leaflet with predominant phospholipids are phosphatidylethanolamine and phosphatidic acid (Fig. 6A) [56]. Fig. 6B reveals the presence of intermolecular interaction between the hydrophilic head group and the hydrophobic tail chain. Head group interaction occurs via a hydrogen bond (in green line) between oxygen phosphatidic acid's phosphate group with the hydrogen of phosphatidylethanolamine's amine group. Hydrophobic bonds (in purple line) are important for the intermolecular and intramolecular interaction between fatty acid tails to strengthen the lipid pack. Interdigitated lipids of the outer and inner of the fatty acid chain also observed occurred via hydrophobic bonds (Fig. 6A). The study reveals that one molecule of piperine may interact with six molecules of lipids through the interaction of hydrophobic alkyl and Pi-alkyl interaction. The obtained data is important for designing liposome formulas, especially during the estimation of piperine loaded into liposome structure.

5. Conclusions

The piperine pro-liposome composed of soy lecithin and cholesterol was successfully prepared without involvement of organic solvent or polyol reagent during process to obtained nano to micrometer size of stable pro-liposome. Several critical parameters were identified, including optimum temperature for lipids and active ingredient homogenization; thin-film hydration technique; and selection of sucrose as a suitable carrier agent in the spray drying process. Depending on the aim of therapeutic application, the piperine pro-liposome obtained from the study can be developed in formulation stage, or further developed to obtain uniform nanoparticle size of the liposome.

Author contribution statement

Eri Amalia: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Iyan Sopyan & Norisca Aliza Putriana: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Sriwidodo Sriwidodo: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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