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In Silico Modeling and Empirical Study of 4-*n*-Butylresorcinol Nanoliposome Formulation

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Abstract

A study to incorporate *in silico* modeling with an empirical experiment has been carried out to formulate nanoliposome containing 4-*n*-butylresorcinol as the active ingredient. The *in silico* modeling was performed using molecular dynamics simulation followed by potential energy calculation to understand the dynamics of the liposome assembly and the morphology of the

formed liposome. The empirical experiment was conducted by formulating the nanoliposome using soy lecithin phospholipid formula as suggested by the *in silico* modeling followed by determining its particle size as well as its shape. From their incorporation, it was found that phospholipid molecules with the number of 3200 were selected in formulating nanoliposome containing 4-*n*-butylresorcinol. The result of the nanoliposomes size observation in the modeling of 3200 lipid molecules was 87.01 (± 0.59) nm, whereas the size from the empirical study was 87.65 (± 0.05) nm.

Keywords: 4-*n*-butylresorcinol, formulation, molecular modeling, nanoliposome, simulation.

List of abbreviation: LUV: large unilamellar vesicles, SUV: small unilamellar vesicles, AFA: Adaptive Focused Acoustics™, CGMD: Coarse-Grained Molecular Dynamic, DMPC: dimyristoyl-phosphatidylcholine, DPPC: dipalmitoylphosphatidylcholine, TEM: Transmission Electron Microscopy, PIPC: palmitoyl linoleyl phosphatidylcholine, vDW: van der Waals.

Introduction

Nanoparticle technology has become one of the trending interests in drug delivery system research in the last decade (Boisseau et al., 2011; Puri et al., 2010). Applied technology in nanoparticle formulation can be performed using two different methods, namely top-down and bottom-up methods (Singh et al., 2011; Adami et al., 2006). Drug delivery system improvement using lipid nanoparticle formulation has been developed by arranging phospholipid bilayer fragments as well as liposomes for a diverse administration route in the human body. Lipid nanoparticle formulation provided several advantages such as its low toxicity, which is proved by *in vivo* observation, and its capability to improve the physical stability of the active ingredient in the dosage form formulation (Akbarzadeh et al., 2013). Nanoparticle technology can be applied in the drug formulation with the encapsulation process by several matrices such as nanospheres, nanoliposomes, and nanoemulsions (Martien et al., 2012).

One of the lipid nanoparticle drug delivery systems that have been commonly developed is nanoliposome since it has been reported with several advantages such as increasing the efficacy and therapeutic index as well as improving the drug stability through the encapsulation system (Akbarzadeh et al., 2013). Liposome, a topical dosage form, has been developed in the drug

formulation due to its good penetration into the skin (El Maghraby et al., 2001; El Maghraby et al., 1999). Nanoliposome showed good activity of penetrating the cell wall and intercellular space due to their nano-size, i.e. < 200 nm (Adami et al., 2006). The trend of nanoliposome formulation development increased continuously since the formulation process was reported to play an essential role in drug encapsulation (Wang et al., 2011; Zhao et al., 2009). The physical stability of the dosage form could be maintained by increasing the drug encapsulation and reducing the liposome leakage during the storage (Eloy et al., 2014; Laouini et al., 2012).

The previous study by Shen et al. (2015) had empirically approached the formulation of large unilamellar vesicles (LUV) or small unilamellar vesicles (SUV) liposomes using focused ultrasonic irradiation with the usage of Adaptive Focused Acoustics™ (AFA) (Shen et al., 2015). This applied method was performed at a low temperature without organic solvent and produced liposomes with a size of < 600 nm.

Molecular modeling in this study was described as a computational approach to study the interaction between drug molecules with the liposome phospholipid membrane and to present the whole molecular interaction during the liposome formation. Rissellada and Marink (2009) studied the effect of temperature and membrane composition during the simulation process towards structure and dynamic behavior of the liposome membrane with the diameter of 15-20 nm using Coarse-Grained Molecular Dynamic (CGMD) simulation (Risselada et al., 2009). Another study by Siwko et al. (Siwko et al., 2009), simulated molecular modeling of dimyristoyl-phosphatidylcholine (DMPC) phospholipid used CGMD at 323 K to investigate the interaction between resorcinol and the bilayer lipid membrane.

In this study, 4-*n*-butylresorcinol was used as the drug instead of resorcinol to be applied as the drug model. It has activity as tyrosinase inhibitor for the management of pigmentation disorders, such as the topical treatment of hyperpigmentation (Kolbe et al., 2013). This resorcinol derivative is more stable than resorcinol due to the occurrence of the oxidation process (Love et al., 2003). A double-blind study for melasma with 4-*n*-butylresorcinol was necessary to achieve the hypopigmenting effect, however, 12 adverse events including mild erythema, dryness, peeling, and desquamation have appeared (Khemis et al., 2007). The 4-*n*-butylresorcinol was encapsulated in liposomes to improve the stability and to reduce skin irritation through hydration of the epidermis (Huh et al., 2010; De Leeuw et al., 2009). The stability should be improved by encapsulating the compound into the complex of nanoliposome structure. The molecular modeling

simulation in the initial step of the formulation was important to predict the physical properties of the lipid nanoparticle complex production. The technique of formulation was necessarily developed to produce nanoliposomes with the size of nano (<100 nm) in a short time usage of sonication. It was reported that the heating and sonication processes were important in the nanoliposomes formulation. Mozafari et al. reported that the production of nanoliposomes with the size of ± 600 nm was successfully carried out by the heating process without any extrusion process in polycarbonate membrane as well as sonication (Mozafari et al., 2007). Another study reported that the particle size reduction (≤ 100 nm) occurred with an increase of sonication time with more than 21 minutes (Silva et al., 2010). Hence, it is important to consider the correlation between the molecular modeling approach and empirical observational results in integrated research. This study aims to correlate a molecular modeling and an empirical observation in the production of nanoliposomes using soy lecithin phospholipid and 4-*n*-butilresorcinol as the delivery system and active ingredient, respectively.

Materials and Methods

1. Materials

Soy lecithin phospholipid (Pharmaceutical Grade, Nacalai Tesque, Jepang), redistilled water, 4-*n*-butylresorcinol (Pharmaceutical Grade, SHREEJI Pharma International, India), ethanol (Analytical Grade, Merck, USA).

2. Gas Chromatography-Mass Spectrometry Analysis (GC-MS) of Soy Lecithin

The GC-MS analysis was performed using a Shimadzu GCMS-QP2010S with Agilent HP-5ms column (30 m x 0.25 mm x 0.25 μm). The injector temperature was set to 310^{o} C. Helium was used as carrier gas at a constant flow rate of 0.40 mL/min. The column temperature was kept at 120^{o} C for 5 min and then increased from 120^{o} to 300^{o} C at 5^{o} C/min. As for the ion source, the temperature was set to 250^{o} C, and the interface temperature was set to 305^{o} C. The mass scanning was set from m/z 28 to 600.

3. Molecular Dynamics Simulation of Soy Lecithin

Coarse-grained molecular dynamics using MARTINI 2.0 force field were used to study the liposome formation of soy lecithin. Prior to the initial coordinate preparation, the coarse-grained

structure of phospholipids (DPPC, POPC, and PIPC), cholesterol, and 4-*n*-butylresorcinol were prepared. The GC-MS analytical result was used to determine the composition of soy lecithin and to construct the initial coordinates according to the composition. The initial coordinate was prepared by a random placement of phospholipid and cholesterol in a 22 x 22 x 22 nm^3 box using the gmx insert-molecule module of GROMACS 2019.1 package on Centos 7.4. Three concentrations of lipids were used i.e. 2400, 2800, and 3200 lipids/boxes. Each concentration of lipid was replicated two times. One of them has not been added by 4-*n*-butylresorcinol while the other one has been added by 4-*n*-butylresorcinol in the molecule numbers of 123, 143, and 164 molecules for 2400, 2800, and 3200 lipids, respectively. In total, there are six different initial coordinates which replicated two times. After the lipid was randomly placed, the box configuration was edited using a gmx edit conf module and solvated with 78.299 water beads using a gmx solvate module generating a system consisted of lipids and water with the size of 24 x 24 x 24 nm^3. The initial coordinates were then subjected to energy minimization using the gmx mdrun module with the steepest descent method. The resulting minimized system then simulated under NPT condition for 240 ns using 40 fs time step, 323 K temperature, and reaction-field long-range electrostatic treatment. The temperature control was using the V-rescale method while the pressure control used the Berendsen barostat. The MD results were then processed with gmx energy module to generate the potential energy data and gmx gyrate module to generate the radius of gyration data. The data were then plotted using Grace. The trajectory file is visualized using VMD 1.9.3 to understand the dynamic of the liposome assembly and the morphology of the liposome.

4. Empirical Observations

The formulation of nanoliposomes with and without 4-*n*-butylresorcinol was carried out by dispersing an amount of soy lecithin over redistilled water using the heating method incombined with sonication. The formula was composed of soy lecithin and other ingredients, as presented in Table 1. The empirical observation was approached by formulating the nanoliposomes using the theoretical data of soy lecithin through molecular modelling of 3200 phospholipid molecules. The phospholipid being used in this study was soy lecithin having transition temperature within 50-60^0C (De Leeuw et al., 2009). The temperature in the formulation was selected at 50° C alongside with the sonication for 30 minutes and 37 kHz of a bath sonicator. Soy lecithin was dispersed in 100 ml of redistilled water at 50°C until the nanoparticle system met the requirement of a

polydispersity index (< 0.3). The selected temperature at 50° C being used during the formulation was performed to approach the temperature condition close to the molecular modeling parameter. The soy lecithin dispersed solution was then blended at a high speed. This aimed to minimize the bilayer fragment sheets that have been formed. The solution being formed was then homogenized using Ultraturac® for 1 minute at four scales and sonicated for a further 30 minutes (Dwiastuti et al., 2016; Dwiastuti et al., 2016) until the particle with <100 nm in size were observed. The product was then left to cool down at room temperature before a morphological as well as the particle size testing. After that, the product was stored in a refrigerator at 4-8°C.

Table 1. The nanoliposome formula on the empirical observation.

Materials	Amount
Soy lecithin	7.74 g
4-*n*-butylresorcinol	0.1% w/v
Redistilled water	100.00 mL

5. The conversion of soy lecithin from molecular modeling into empirical observation.

The soy lecithin being used in the empirical observation was based on the number of phospholipid molecules in nanoliposomes modeling. The concentration of phospholipid in the simulation of 3200 phospholipid molecules was 387.12 mg/mL, which equals to 0.38712 g/mL.

The amount of soy lecithin being used: 0.38712 g/mL x $\frac{\text{the box size in modeling}}{100\ \text{nm}}$

$$: 0.38712\ \text{g/mL} \times \frac{(20\text{nm})}{100\ \text{nm}}$$

$$: 0.077424\ \text{g/mL} \sim 7.74\ \text{g}/100\ \text{mL}$$

According to the conversion above, therefore, the soy lecithin being used here was 7.74 g in 100 mL of redistilled water.

6. Determination of the particle size

The determination and distribution process in the system was conducted using particle size analyzer instrument, Horiba SZ-100, based on the scattering dynamic light principle with temperature of 24.8-25.0°C and angle of 90°. It was 0.50 µL of the sample transferred into a 25 mL of a volumetric flask, and then added by redistilled water up to the calibration sign. A volume of 2.00 mL of aliquot was transferred into cuvet for the measurement of 100,000 particles.

7. Determination of a lamellar particle

The observation of a lamellar particle used Transmission Electron Microscopy (TEM) JEM-1400 (Faculty of Natural Sciences, Gadjah Mada University). The determination of the lamellar particle was carried out to lipid nanoparticle with and without the presence of 4-*n*-butylresorcinol. A volume of 0.5 mL of the formula was added with 1 mL of redistilled water, and then dropped into an object plate. The electromagnetic transmission light was subjected to the plate, and then the particle morphology was observed at a suitable magnitude. Morphological results of the sample were displayed on the camera screen with no staining process. The result was then visualized as the lamellar particle shape.

Results and Discussion

The GC-MS of Soy Lecithin

The soy lecithin contents were analyzed using GC-MS method. The retention time, percentage of peak area, and the base peaks as well as their interpretation were furtherly detailed and presented in Table 2.

Table 2. GC-MS analysis result of the soy lecithin

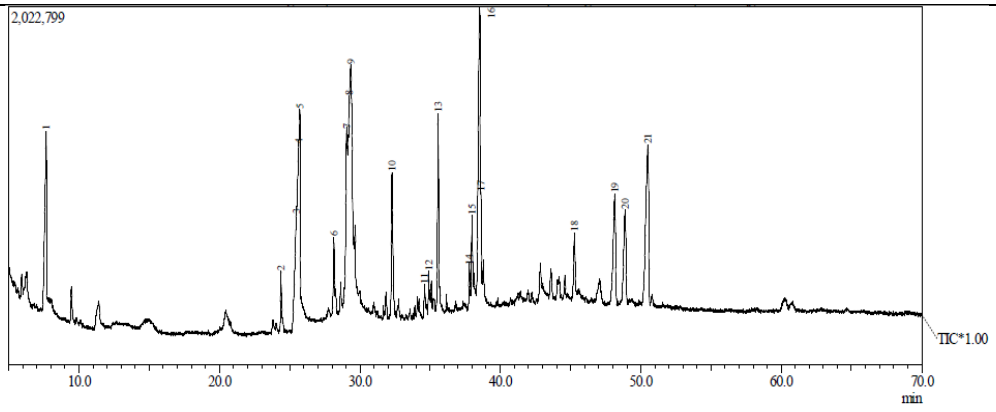


Table 1. GC-MS

No	Peak #	Retention Time (minute)	Area (%)	Base peak (MW)	Interpreted as (MW)
1	2	24.38	1.49	74.05	Methylpalmitate (270)
2	5	25.73	5.42	43.10	Palmitic acid (256)
3	6	28.15	1.50	55.05	Methyl oleate (296)
4	9	29.35	11.17	55.10	Linoleic acid (280)
5	13	35.58	5.25	43.05	Glycerol 1-palmitate (330)
6	16	38.52	14.09	55.10	2-hydroxy-1-(hydroxymethyl)ethyl ester linolein (354)
7	18	45.28	1.89	43.10	Cholesteryl myristate
8	19	48.13	4.85	43.05	5-ergostenol (400)
9	20	48.90	3.98	55.10	Stigmasterol (412)
10	21	50.49	10.09	43.05	Clionasterol (414)

Note: only he lipids and positive compound are shown

In general, it was found that there were two primary compounds in soy lecithin namely fatty acid and sterol (Table 3). The detected fatty acids were recognized as palmitic acid, oleic acid, and linoleic acid; whereas, the sterol contents were recognized as clionasterol, 5-ergosterol, and stigmasterol.

Table 3. Soy lecithin primary component profiles

Component	Peak #	% Area	Total % Area
Fatty acid			
Palmitate	2, 5, 13	1.49 + 5.42 + 5.25	12.16
Oleate	6	1.50	1.50
Linoleate	9, 16	11.17 + 14.09	25.26
		Total fatty acids	38.92
Sterol			
5-ergostenol	19	4.85	4.85
Stigmasterol	20	3.98	3.98
Clionasterol	21	10.09	10.09
		Total sterol	18.92

From Table 3, it should be noted that only several lipids content were detected and calculated by the GC-MS method due to their thermal instability during analytical process. Therefore, the percentage of total area calculation was still below 100%. It can be assumed that palmitate content resulted in 54.32% since palmitate was found as major compounds in soy lecithin. Finally, the number of molecules for each lipid can be determined by multiplying the percentage and the total lipid, which can be seen in Table 4.

Table 4. Number of lipid molecules for each system in molecular modelling simulation

System #	N of lipids	DPPC	POPC	PIPC	Cholesterol
1	2400	1304	36	606	454
2	2800	1521	42	707	530
3	3200	1738	48	808	606

Note. DPPC = dipalmitoyl phosphatidylcholine; POPC = palmitoyl oleyl phosphatidylcholine; PIPC = palmitoyl linoleyl phosphatidylcholine

The Potential Energy Calculation

The temperature adjustment was set to 50° C during the simulation due to the protocol published in the previous study by Siwko et al. (2009). Figure 1 depicted the graphical plot of potential energy-time dependent occurred during the simulation of 2400, 2800, and 3200 lipid molecules. The energy calculation involved in this study during the simulation processes were kinetic and potential energies.

Tabel 5. The comparison between the time, the potential energy, and the shape of liposome being formed during the simulation with the number of lipids and 4-*n*-butylresorcinol treatment variation.

N lipid	*n*-butyl resorcinol	T enclosed (ns)	E1 (x10^6 kJ/mol)	E2 (x10^6 kJ/mol)	E3 (x10^6 kJ/mol)	Shape
2400	No	90	-2.947	-2.962	-2.963	Rod
		19	-2.942	-2.948	-2.952	Spherical
	Yes	56	-2.954	-2.968	-2.969	Elliptical
		31	-2.947	-2.953	-2.957	Spherical
2800	No	40	-2.941	-2.949	-2.951	Spherical with torus-shaped cavity
		20	-2.948	-2.956	-2.959	Spherical
	Yes	68	-2.947	-2.957	-2.959	Elliptical with U-shaped cavity
		18	-2.950	-2.955	-2.958	Spherical
3200	No	12	-2.937	-2.939	-2.945	Spherical
		24	-2.939	-2.941	-2.945	Spherical with crescent-shaped cavity
	Yes	32	-2.945	-2.953	-2.955	Spherical with torus-shaped cavity
		14	-2.950	-2.954	-2.958	Spherical

T = The time until liposome fully enclosed
E1 = The energy at 10 ns
E2 = The energy when liposome fully enclosed
E3 = The energy at 240 ns

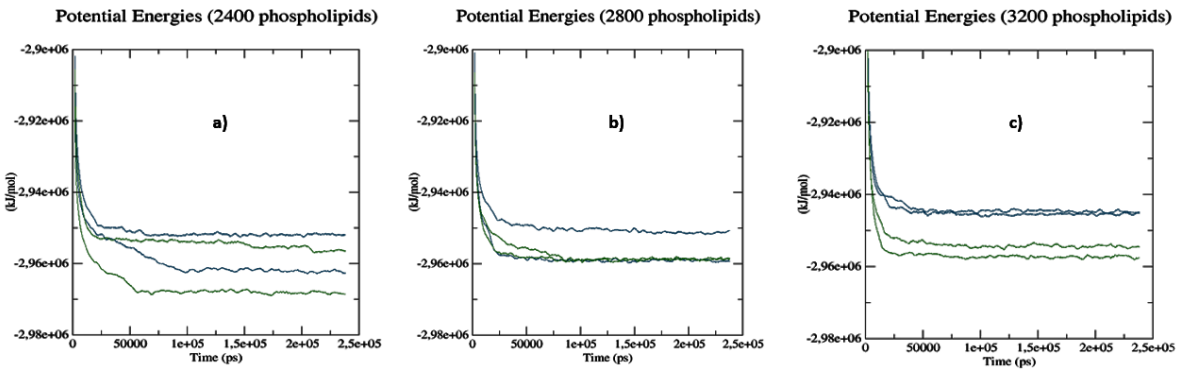


Figure 1. The graph plotting potential energy-time dependent occurred during the simulation of a) 2400, b) 2800, and c) 3200 lipid molecules. Note: Simulation without 4-*n*-butylresorcinol has been shown by blue lines, while simulation with 4-*n*-butylresorcinol has been shown by green lines.

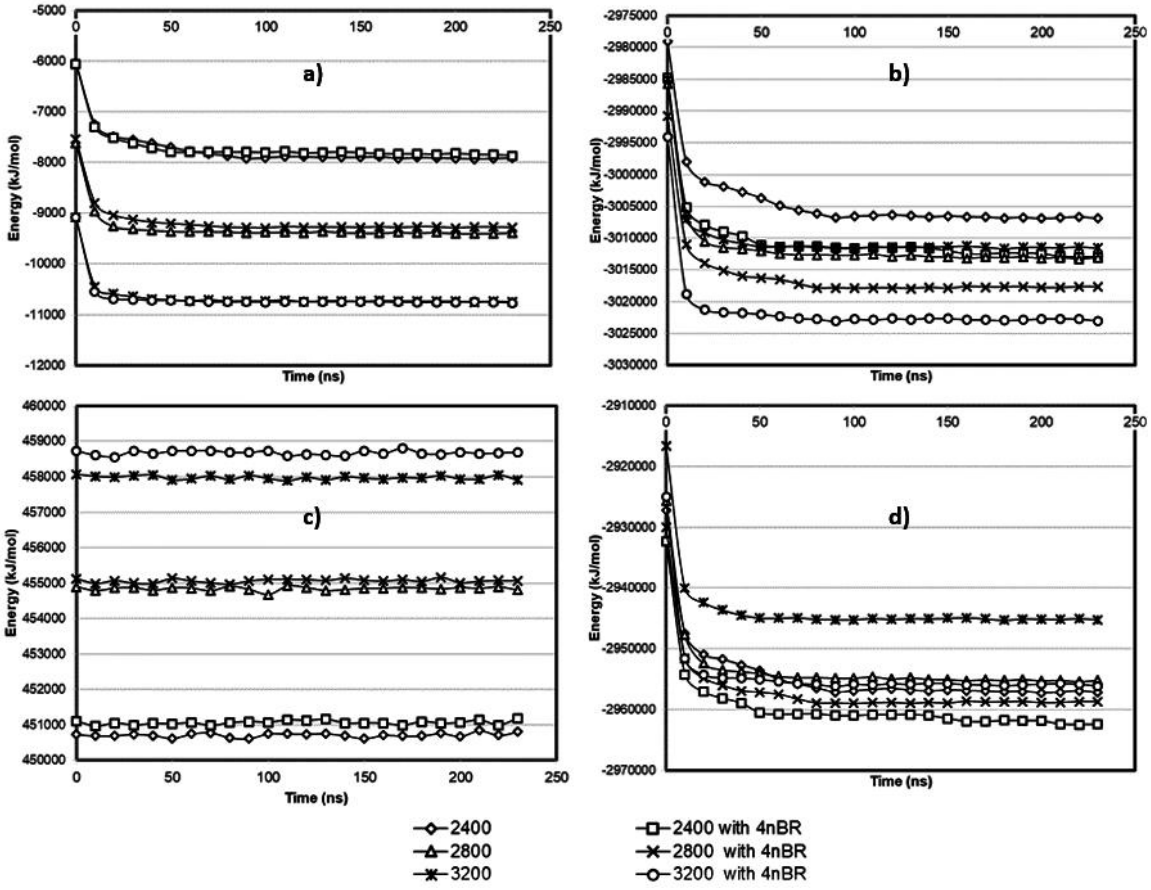


Figure 2. The graph plotting potential energy-time dependent occurred during the simulation of a) electrostatic, b) vDW, c) kinetic, and d) total energy.

The Radius of Gyration of Molecular Modeling Simulation

The radius of gyration can be described as a unit for the radius average from the molecule to the centre of mass to determine the liposome diameter. The radius of gyration of nanoliposome was formed during the simulation of 2400, 2800, and 3200 lipid molecules with and without the presence of *4-n*-butylresorcinol (Figure 3). The nanoliposomes morphological results of the simulation of 2400, 2800, and 3200 lipid molecules and their snapshots of nanoliposomes formation results during simulation can be seen in Figure 4 and Figure 5, respectively.

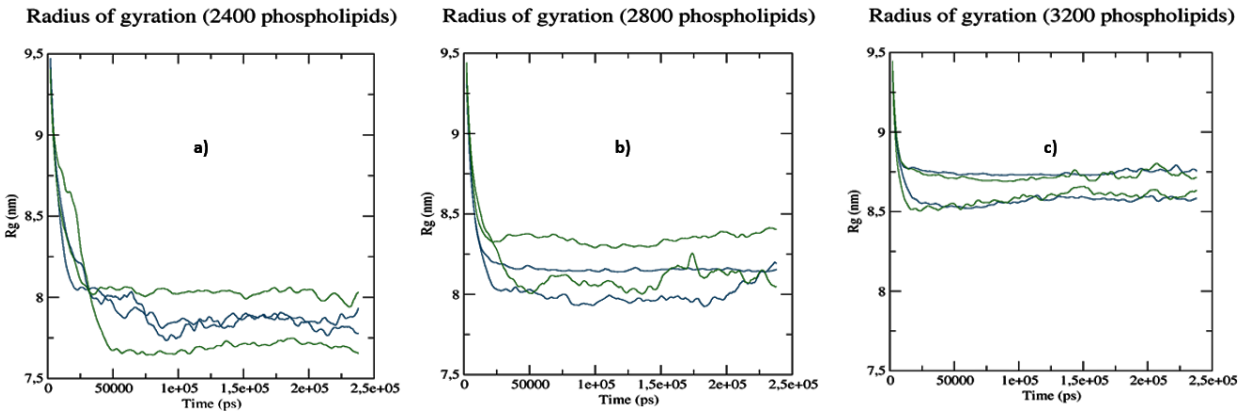


Figure 3. The gyration radius of the nanoliposomes being formed during the simulation of a) 2400, b) 2800, and c) 3200 of lipid molecules with (green) and without (blue) the presence of *4-n*-butylresorcinol.

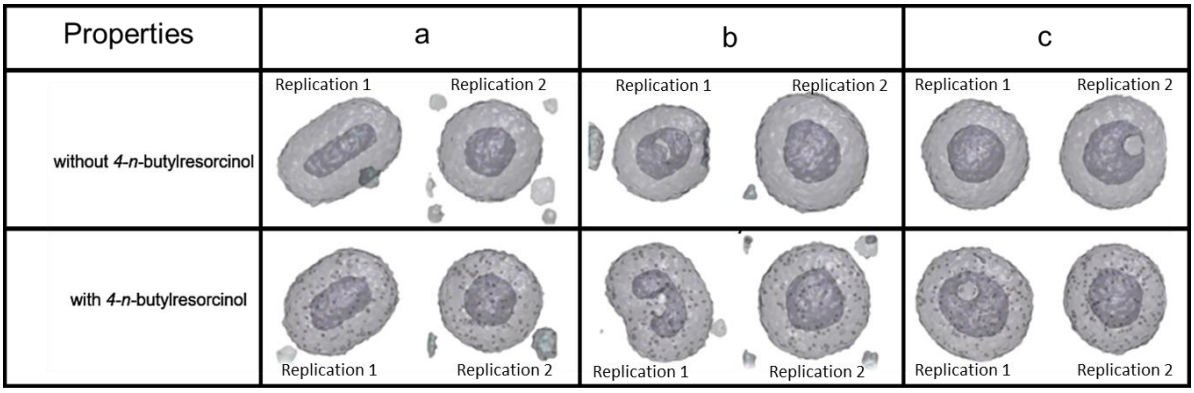


Figure 4. Nanoliposomes morphological results of the simulation of a) 2400, b) 2800, and c) 3200 lipid molecules with two replications for each lipid molecule number.

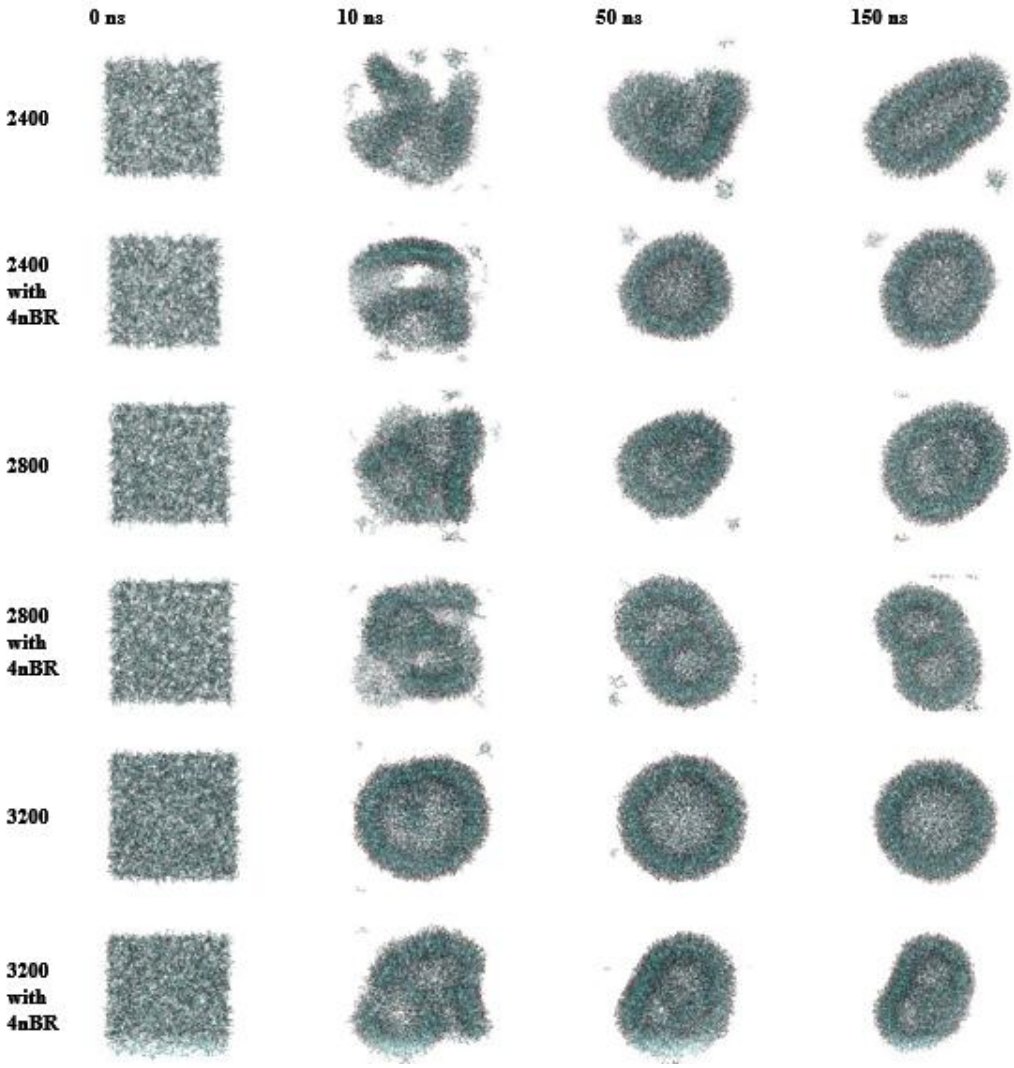


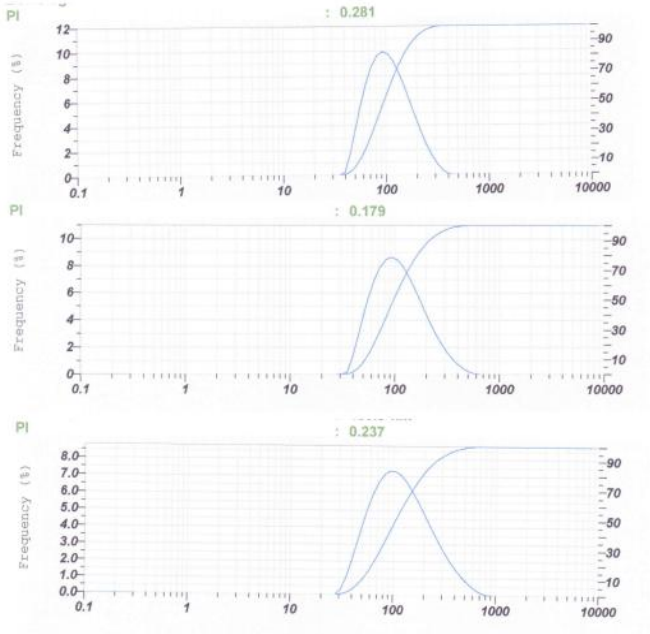
Figure 5. Snapshots of nanoliposomes formation results during the simulation

The Size of Nanoliposome with The Presence of 4-*n*-Butylresorcinol

The size of nanoliposome resulted from both molecular dynamics simulations and empirical observations were compared and presented in Table 6.

Table 6. Results of nanoliposome size based on simulation and empirical observation

Nanoliposome sizes based on the in silico simulations (3200 molecules)		Nanoliposome sizes based on the empirical observation		
Replication	**Nanoliposome sizes (nm)**	**Replication**	**Calculation mode (nm)**	**Polydispersity Index**
1	86.60	1	87.60	0.28
2	87.43	2	87.50	0.18
		3	87.60	0,24
Mean (±SD)	87.01 (± 0.59)	Mean (±SD)	87.57 (± 0.06)	



The Morphology of 3200 Phospholipid Molecules

The morphological observation of 3200 phospholipid molecules from the simulation model was compared to the empirical observation without the presence of 4-*n*-butylresorcinol, which can be seen in Figure 6, while the interaction between drug and the membrane model was depicted in Figure 7.

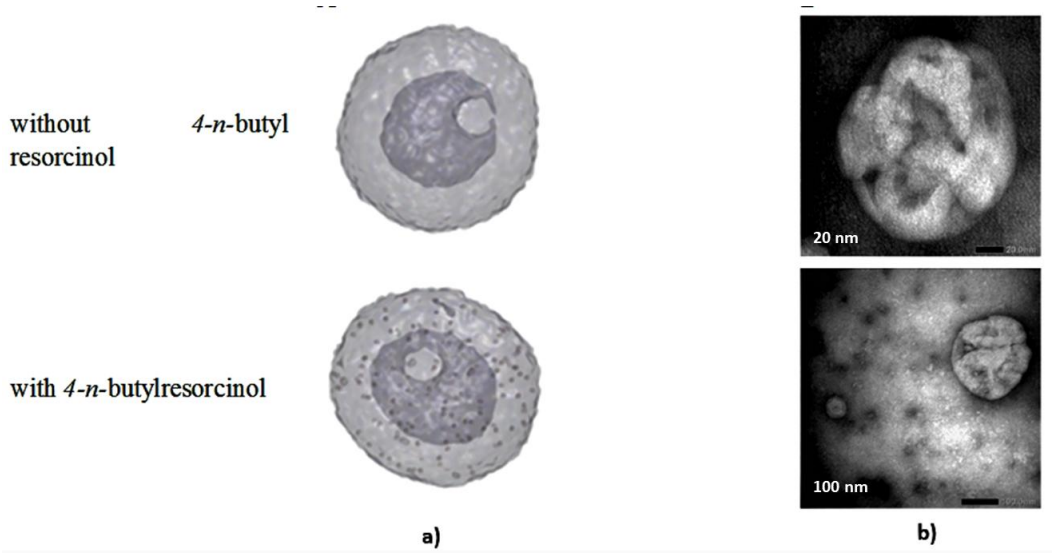


Figure 6. The nanoliposome morphology being formed from a) the modeling and b) the empirical observation on 3200 lipid molecules.

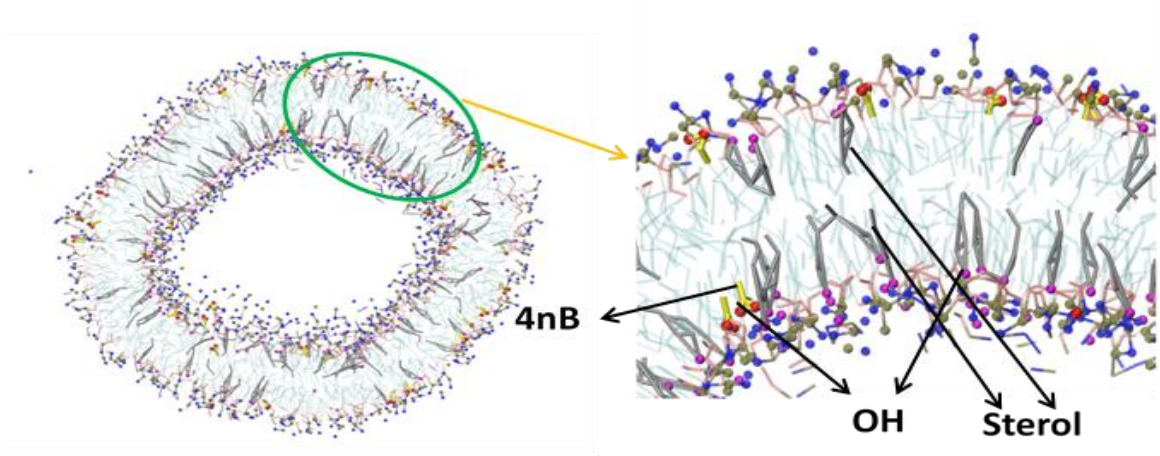


Figure 7. The interaction of the model compound in the lipid membrane

The GC-MS method was utilized to determine the chemical composition of the soy lecithin as listed in Table 2. The fatty acids of the phospholipid and sterol were used as the platform in the nanoliposomes molecular modeling. The identified compositions of fatty acid from the GC-MS were palmitic acid, oleic acid, and linoleic acid. It is appropriate with the composition of soy lecithin theoretically (Perkins et al., 1995; Van Hoogevest et al., 2013). The sterol being composed in the soy lecithin was 5-ergosterol, stigmasterol, and clionasterol (Table 3). The percentage of fatty acid and sterol as calculated in Table 3 was used as the basic calculation to decide the composition of the nanoliposomes modeling.

The nanoliposomes modeling with and without the presence of 4-*n*-butylresorcinol used three variations of the lipid molecule number, i.e. 2400, 2800, and 3200 molecules. This observation was aimed to predict the possibility of the nanoliposomes formation with and without the presence of 4-*n*-butylresorcinol by molecular modeling on a few lipid molecules being used. The decision of selecting the number of lipid molecules was applied and referred to the study from Koshiyama and Wada (2016). The results indicated a formation of nanoliposomes from modeling using 2400, 2800 and 3200 molecules inside 22 nm^3 of the box size (Figure 2).

According to the graphical energy presented in Figure 1 and 2, in general, it was showed that the potential energies were stronger for a lower lipid number. This trend appears as the lower number of lipids, the higher number of waters, which contributes to the stronger electrostatic and vDW interactions. Whilst in term of the velocity and its stability, both the energy graph as well as the radius of gyration graph, shows that at a higher number of lipids, the liposomes were formed faster and quickly stabilized. It is also important to note that the slower liposome formation tends to give the phospholipid bilayer a chance to form a multiple connection, leading to an imperfect sphere or a bilayer bridge in the middle of the liposome. Therefore, the 3200 lipids molecule was subjected for the empirical nanoliposomes formulation by with and without the presence of 4-*n*-butylresorcinol. The formation of nanoliposomes in the molecular modeling involves kinetic and potential energies in the form of electrostatic and vDW interactions. According to the energy calculation results in molecular modeling simulation at 50°C, the nanoliposome system was stable at about 100 ns (Figure 1 and Figure 2). Nanoliposome structures were generated in the molecular modeling simulation with the electrostatic energy. It may be resulted from the interaction between electronegative atoms in the phosphate group of the phospholipid with the hydrogen atom from the water molecules around the system. Furthermore. an electrostatic interaction between polar groups of phospholipid during simulation also occurs. The lowering of vDW energy indicated a similar graphical profile with the lowering of electrostatic and total energies in the system. The interaction between nonpolar group during the simulation resulted vDW energy. With the longer simulation time, the electrostatic, vDW, and the total energy were decreased due to the system stabilization in order to achieve the nanoliposomes structure (Figure 5) for simulation, with and without *4-n*-butylresorcinol in three different molecules number (2400, 2800, and 3200). The system stabilization has been achieved when the unilamellar nanoliposomes was formed in 100 ns. Moreover, the electrostatic, vDW, and total energies were shown to be decreased in the both system with and without *4-n*-butylresorcinol during the simulation. The vDW energy depicted interactions between nonpolar groups of phospholipid molecules and nonpolar group of *4-n*-butylresorcinol. Chemical properties of the nonpolar model compound might affect the vDW interaction at the initial and at the end of the simulation. The interaction between 4-*n*-butylresorcinol-lipid and lipid-membrane during the simulation process resulted similar characteristic results with the previous study (Siwko et al., 2009), where the hydroxyl (-OH) group of *4-n*-butylresorcinol interacted with the hydrophilic group of the phospholipid compound (Figure

7). The presence of *4-n*-butylresorcinol increased the vDW interaction between non polar groups, whereas the electrostatic interaction in phosphate group with hydrogen atom from water was decreased since there were several hydrogen bonding between two phospholipid molecules. The morphological results of the molecular modeling simulation (Figure 5) indicated that nanoliposomes was formed into unilamellar structure. Molecular modeling simulation with 3200 phospholipid molecules resulted a similar form of nanoliposomes compared to the empirical observation result (Figure 6).

The conversion of the phospholipid from the simulation was done using a concentration in that 3200 molecules by 0.38712 g/mL with the conversion factor = 0.2. This factor was a ratio between the box size used in the simulation (20 nm) and the actual box should be used in the empirical observation, i.e. 100 nm. The parameter being observed in the nanoliposomes product was the size and the morphology of the nanoliposomes being formed. The result of the nanoliposomes size in the molecular modeling of 3200 lipid molecules was 87.01 nm (± 0.59), whereas the size from the empirical study was 87.65 nm (± 0.05). These results indicated that there were no significant different for the size and the morphology of the nanoliposomes formed with and without the presence of 4-*n*-butylresorcinol, which found a deal between these *in silico* and *in vitro* studies.

Conclusions

Molecular dynamics simulation was proved as the tool that is strongly predicting the dynamics behavior of 4-*n*-butylresorcinol upon nanoliposome formulation. The program was able to predict the number of phospholipid molecule during the formulation which resulted in agreement between *in silico* and empirical study. It was found that phospholipid molecules with the number of 3200 were selected in formulation process of nanoliposome containing 4-*n*-butylresorcinol. The molecular modeling and empirical studies resulted the molecules size of 87.01 (± 0.59) and 87.65 (± 0.05) nm, respectively. These significant results indicated the deal between the molecular modeling and the empirical observation in formulating nanoliposome.

Acknowledgments

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Disclosure statement

No potential conflict of interest reported by the authors.

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Your manuscript entitled "In Silico Modeling and Empirical Study of 4-n-Butylresorcinol Nanoliposome Formulation" has been successfully submitted online and is presently being given full consideration for publication in Journal of Biomolecular Structure & Dynamics.

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05-Mar-2021

Dear Dr Dwiastuti:

Your manuscript entitled "In Silico Modeling and Empirical Study of 4-n-Butylresorcinol Nanoliposome Formulation", which you submitted to Journal of Biomolecular Structure & Dynamics, has been reviewed. The referee comments are included at the bottom of this letter.

The referee(s) would like to see some revisions made to your manuscript before publication. Therefore, I invite you to respond to the referee(s)' comments and revise your manuscript.

When you revise your manuscript please highlight the changes you make in the manuscript in yellow high light. Please also ensure that you submit a clean version using the file designation 'Manuscript with track changes removed.'

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Sincerely,
Professor Sarma
Editor in Chief, Journal of Biomolecular Structure & Dynamics
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Referee(s)' Comments to Author:

Referee: 1

Comments to the Author
Abstract:
Please mention which type of MD simulation was performed in your study.
It is confusing the way in which it is described how MD simulation assist in nanoliposome formulation .
Introduction
Page 3, line 70-77. This paragraph is part of the methodology? it is not clear why this paragraph is placed here. So, I recommend you to cite this text and showed some examples.
Page 3, line 79-80. please put the reference

Page 3, line 85-87. Page 4, line 96-970. You describe a study related to the field, however it is not clearly explained why is the relation with your study or why did you include this citation, please describe the relation with your study or why it was used as background.
Along the manuscript you mentioned molecular modeling, but the concept is employed under different scenarios, please clarify the concept because it is misused in some of them.
Page 4, line 103. it is advisable to mention the time of the application, because relatively in terms of clinical studies its is imprecise and it is not a common term.
Page 4, line 107-108. You mention that the stability is improved by encapsulating the compound, but is this a rule? Please add the reference.
Along the manuscript you mix terms like molecular modelling with Molecular dynamic simulations, and they are different tools of in silico studies. Please clarify the concepts and use them properly in the text.
I recommend you to rewrite your introduction because since my point of view you stat each paragragh with some lines of your work (metodology) followed by literature that support the the initial idea. it results very confusing to follow the idea that you want to transmit, it is very hard to understand the background of your work, besides I recommend you to add the reference where correspond because there are several ideas without references.
Material and methods
In section 2., GC-MS analysis, I suggest you to add a couple of line clarifiying why did you performed GC. Please also mention which is the ion source?
Why did you use GC and not other thectnique in ofrder to avoiod the thermal insability mentioned in the result section???
In section 3. Molecular dynamics simulation. I recommend you to menione how di you determine the composiion of the liposome at the beginning of the paragraph.
Please also give the number of water beads used.

Please clarify the temperature used in your smulation and experiments, because in MD section you mentioned that it was carried out at 323 K , and in the experimental section you say that you employed 500 °C, in kelvins it is 773.15 K.
Section 5. The conversion of soy lecitin
did you get the concentration of tthe phospholipids in your sistyem 387.12 mg/mL
Please review the calculation performed in the section five, since there are mistakes in the numbers.
Results
The potential energy calculation
the term "the number of potential energy" is not correct, please verify it.
page 10. the phrase "In contrast, the increase of the lipid number, the decrease of
244 the hydrogen bond in water", Please clarify the sentence because it is not clearly explained to what are you referring to.
Table 5. Please describe which type of energy is showed in this table.
Figure 1. Why are there four lines per graphic? please clarify
Figure 2. Please describe these graphs and what did you conclude about them?
Page 12. You write: The radius of gytation of nanoliposome was formed....
Please clarigy because the Rg was not formed, the nanoliposopme was formed and the Rg allow you to measure the nanoliposome formation.
I suggest you to be more descriptive about your MD simulation.
Figure 4. Why did you put a couple of figures per section. It would be better if you use boxes to separate these figures.
pag. 16, The phrase "structures were generated in the molecular
332 modeling simulation without the electrostatic energy", how did you omit the electrostatic energy?? Please clarify
It seems that the basis of molecular dynamic simulation are not clear, such as potential energy and the components of the same. Please review this concepts in order to improve the discussion of your results.

Referee: 2

Comments to the Author
Manuscript ID: TBSD-2021-0257

Reviewer´s comments:
The manuscript entitled “In Silico Modeling and Empirical Study of 4-n-Butylresorcinol Nanoliposome Formulation”, describes the molecular dynamics simulations of three lecithin systems with different numbers of molecules for liposome formation. One of these systems was prepared experimentally and it was confirmed that the theoretical and experimental liposome sizes coincide.

As a general comment, this article is relevant because using soy lecithin in liposomal formulations reduces costs considerably, compared to using pure phospholipids. The authors' proposal to carry out soy lecithin molecular dynamics (MD) experiments in order to predict the size of liposomes is a very good idea and improves the liposome formation methodology.
However, the authors do not discuss the differences or similarities of liposomes obtained by MD with and without 4-n-butylresorcinol (size, energy and shape). They only give the theoretical size of one of the systems, but it would be necessary to give the sizes of the 2 missing systems to verify if the difference is relevant or not and include some discussion. The manuscript can be accepted after addressing the following comments.

1. Page 1. Title: The author uses the word “Nanoliposom” instead of “Nanoliposome”. Please change.
2. Page 2 line 70: The information can be expanded by mentioning physicochemical parameters as size and zeta potential. They are important for the stability.
3. Page 4 line 94: The authors didn´t mention the results found by the aforementioned researchers.
4. Methodology: the authors must clarify if the liposome size reported is the radius or the diameter. It is desirable that they clearly explain how the liposome size is calculated from the radius of gyration.
5. Page 5 line 151: Concentration of 4-n- butylresorcinol was not given.
6. Page 6 line 172: the authors say that “The temperature in the formulation was selected at 500 °C ...” Please correct.
7. Page 7 line 200: The angle and the temperature used in the liposome size measurements are not given. It should be clarified if the size distribution was measured by intensity, number or volume, since choosing one of these parameters over the others give variations in size.
8. Figure 1 y 3: The authors didn´t explain why there are 2 blue lines and 2 two green lines. Please explain what each means. The chosen colors are difficult to distinguish.
9. Figure 4: Why they put two images for each system? Please clarify.
10. Table 6: Please add the liposome sizes found for all systems studied by MD. (2400 an 2800 are missing).

11. Figure 6: Specify the size of the scale bar in the caption of figure 6, since it is not visible in the photo. The authors should mention the diameter measurement of the liposomes shown in figure 6 by TEM.
12. The authors should include the liposome size distribution graphs obtained from the DLS, to see if there is a single population or not.
13. Do the number of drug molecules affects the liposome size?. Please clarify.
14. It is desirable that the authors perform the experiments with the 3200 system without drug in order to corroborate their MD results. Also mention how reproducible is their experimental procedure, it seems that they only made once the experiments.
15. Page 16 line 324: The authors say “It is also important to note that the slower liposome formation tends to give the phospholipid bilayer a chance to form a multiple connection, leading to an imperfect sphere or a bilayer bridge in the middle of the liposome.” Please mention the corresponding figures.
16. Page 17 line 374: The authors say “These insignificant results ….” I guess the authors didn´t mean insignificant, please verify. I´m not sure what do the authors mean by “deal”. Could you please clarify?

Referee: 3

Comments to the Author
1.The idea of the work is good but 80% of the work is based on computational analysis it lacks experimental evidence so requires a few experimental data to validate it

Editor's Comments to Author:

Associate Editor
Comments to Author:
(There are no comments.)

Outlook

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Dear Dr Dwiastuti:

Your manuscript entitled "In Silico Modeling and Empirical Study of 4-n-Butylresorcinol Nanoliposome Formulation" has been successfully submitted online and is presently being given full consideration for publication in Journal of Biomolecular Structure & Dynamics.

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IN SILICO MODELING AND EMPIRICAL STUDY OF 4-N-BUTYLRESORCINOL NANOLIPOSOME FORMULATION

-- Response to the reviewers' comments –

Reviewer 1

No	Comments and Responses
1	**Comments:** Page 3, line 70-77. This paragraph is part of the methodology? it is not clear why this paragraph is placed here. So, I recommend you to cite this text and showed some examples. **Responses:** This paragraph is not part of the methodology. It describes how important the computational prediction on how nanoliposome material will effectively interact with the drug (active ingredient) at molecular or atomic level before the experimental formulation will be performed, so that the formula composition could be rationalized. As stated in the paragraph, this computational prediction can reduce the trial-and-error problems such as time-consuming process, organic solvent selection, and the cost of production. This description is part of the background of our study.
2	**Comments:** Page 3, line 79-80. please put the reference **Responses:** Thank you for the suggestion. We have put some references regarding the success of in silico modeling in nanoliposome formulation Dogra, P., Butner, J. D., Chuang, Y. L., Caserta, S., Goel, S., Brinker, C. J., ... & Wang, Z. (2019). Mathematical modeling in cancer nanomedicine: a review. Biomedical Microdevices, 21(2), 1-23. Cern, A., Marcus, D., Tropsha, A., Barenholz, Y., & Goldblum, A. (2017). New drug candidates for liposomal delivery identified by computer modeling of liposomes' remote loading and leakage. Journal of Controlled Release, 252, 18-27. Cern, A., Barenholz, Y., Tropsha, A., & Goldblum, A. (2014). Computer-aided design of liposomal drugs: in silico prediction and experimental validation of drug candidates for liposomal remote loading. Journal of Controlled Release, 173, 125-131.
3	**Comments:** Page 3, line 85-87. Page 4, line 96-970. You describe a study related to the field, however it is not clearly explained why is the relation with your study or why did you include this citation, please describe the relation with your study or why it was used as background. Along the manuscript you mentioned molecular modeling, but the concept is employed under different scenarios, please clarify the concept because it is misused in some of them. **Responses:** Page 3, line 85-87. Thank you for the suggestion. We agree to remove this citation as this part seems irrelevant with the paragraph's topic.

	Page 4 line, line 96-97. Thank you for the suggestion. This statement giving another example on the use of in silico study using coarse grain molecular dynamic (GGMD) simulation which should be applied in its empirical study. We have added some further explanation regarding with the lack information on how the previous study done by Hudiyanti et al., (2014). In this study, three types of liposome (origin, deformed liposome, and planar bilayer) were in silico simulated to interact with water to give an insight understanding which type of liposome will give the most stable structure during 160 ns of the simulation time. Therefore, the use of CGMD in drug delivery system modeling has been commonly used to provide the best formula in the liposome production. The concept here is to proof the in silico modeling of nanoliposome formulation by studying the atomic interactions between the drug (4-n-butylresorcinol) and the soy lecithin phospholipid based on their composition using molecular dynamic (MD), will aid the selection on nanoliposomes formula more accurately than by trial and error. The result proves that the selected composition provides the close values of nanoliposome size between in silico (87.01 ± 0.59 nm) and empirical study (87.65 ± 0.05 nm) confirming that the in silico modeling strongly guides the empirical study in nanoliposome formulation. To avoid the confusedness, we make the terminology to be more consistent by using "in silico modeling' than molecular modeling.
4	**Comments:** Page 4, line 103. it is advisable to mention the time of the application, because relatively in terms of clinical studies its is imprecise and it is not a common term. **Responses:** Thank you for the advices. We confirm that the 4-n-butylresorcinol time of application clinically is twice daily within 8 weeks. We have added this information in Page 4 line xxx.
5	**Comments:** Page 4, line 107-108. You mention that the stability is improved by encapsulating the compound, but is this a rule? Please add the reference. Along the manuscript you mix terms like molecular modelling with Molecular dynamic simulations, and they are different tools of in silico studies. Please clarify the concepts and use them properly in the text. I recommend you to rewrite your introduction because since my point of view you stat each paragragh with some lines of your work (metodology) followed by literature that support the the initial idea. it results very confusing to follow the idea that you want to transmit, it is very hard to understand the background of your work, besides I recommend you to add the reference where correspond because there are several ideas without references. **Responses:** Thank you for the suggestion. We have added further explanation regarding with the encapsulation rule in polyphenolic compound along with the reference. "Encapsulating phenol-related compounds such as 4-n-butylresircinol aims to preserve the biological activity and to improve the stability of the active compounds, as well as to ensure controlled release of the latter. Encapsulation has the advantage of being a nonthermal stabilization approach, suitable for temperature-sensitive natural biologically active compounds.

	The wall material usually improves the stability of the active compounds by protecting them from direct exposure to air and light (Coradini et al., 2014)". See Page x line xx. Coradini, K., Lima, F. O., Oliveira, C. M., Chaves, P. S., Athayde, M. L., Carvalho, L. M., et al., Co-encapsulation of resveratrol and curcumin in lipid-core nanocapsules improves their in vitro antioxidant effects, Eur J Pharm Biopharm 88 (2014) 178–185. Arthur udah ada di manuscript. We have clarified the concept as responsed in Comment 3. "The concept here is to proof the in silico modeling of nanoliposome formulation by studying the atomic interactions between the drug (4-n-butylresorcinol) and the soy lecithin phospholipid based on their composition using molecular dynamic (MD), will aid the selection on nanoliposomes formula more accurately than by trial and error. The result proves that the selected composition provides the close values of nanoliposome size between in silico (87.01 ± 0.59 nm) and empirical study (87.65 ± 0.05 nm) confirming that the in silico modeling strongly guides the empirical study in nanoliposome formulation" Thank you for the suggestion. The introduction was started by describing the importance of nanotechnology in the drug delivery system to improve its stability during application. One of the delivery system is nanoliposome with phenol-related compound as the active ingredient. The nanoliposome formulation has been broadly conducted, however, most likely the formulation process was carried without mathematical prediction which could lead to the trial-and-error results. The mathematical prediction can be performed using in silico tool such as molecular dynamic which can define the stability of such system in a time-dependent manner. Some published literatures describing the technique in nanoliposome empirical study as well as its in silico study have been employed and cited in introduction, to strengthen our present study in incorporating in silico study and empirical observation in the 4-n-butylresorcinol nanoliposome formulation. We have added references in every single statement when it is corresponded.
6	**Comments:** Material and methods In section 2., GC-MS analysis, I suggest you to add a couple of line clarifiying why did you performed GC. Please also mention which is the ion source? Why did you use GC and not other thectnique in ofrder to avoiod the thermal insability mentioned in the result section??? **Responses:** Thank you for the suggestion. We used the GC-MS as this technique is suitable for lipid identification as the lipids are usually presenting in ester form (Ref). Ester is usually volatile, therefore GC-MS is one of the suitable technique to identify lipid both for its qualitative as well as quantitative prediction (Ref). GC-MS is fast and cheap method, providing the compounds library which could easily predict the identified peak in GC which corresponds to the mass spectrum in the available database. The % similarity in the MS result enhances the power on this instrument to be utilized in our lipid determination. The ion source is electron impact 70 MeV resulting to fragmentation of the parent molecule into its most stable peak (base peak).

	However, this technique has disadvantage as the thermal stability of the compounds becomes the major issue. LC-MS could be the alternative method to reduce this thermal instability, unfortunately, we could not perform this method at the moment due to our limitation. This explanation has been added in Page xx line xx.
7	**Comments:** Material and methods In section 3. Molecular dynamics simulation. I recommend you to menione how di you determine the composiion of the liposome at the beginning of the paragraph. Please also give the number of water beads used. **Responses:** Thank you for the suggestion. We have put the composition determination at the beginning of the paragraph in Page xxx line xxx.
8	**Comments:** Material and methods Please clarify the temperature used in your smulation and experiments, because in MD section you mentioned that it was carried out at 323 K , and in the experimental section you say that you employed 500 °C, in kelvins it is 773.15 K. **Responses:** We have revised the temperature. The correct one is 50°C.
9	**Comments:** Material and methods Section 5. The conversion of soy lecitin did you get the concentration of tthe phospholipids in your sistyem 387.12 mg/mL Please review the calculation performed in the section five, since there are mistakes in the numbers. **Responses:** We have checked and revised this calculation.
10	**Comments:** Results The potential energy calculation the term "the number of potential energy" is not correct, please verify it. **Responses:** We have revised the sentence as follow: The potential energy in the modeling of 2400, 2800, and 3200 phospholipid molecules were calculated.
11	**Comments:** Results Page 10. the phrase "In contrast, the increase of the lipid number, the decrease of 244 the hydrogen bond in water", Please clarify the sentence because it is not clearly explained to what are you referring to. **Responses:** We have remove the sentence to avoid ambiguity.
12	**Comments:** Results

	Table 5. Please describe which type of energy is showed in this table. **Responses:** We have revised the table. The energy mentioned here referred to the potential energy.
13	**Comments:** Results Figure 1. Why are there four lines per graphic? please clarify **Responses:** Di rephrase captionnya.==>DONE
14	**Comments:** Results Figure 2. Please describe these graphs and what did you conclude about them? **Responses:** Buat more explanation of each figures and the conclusion.==> DONE
15	**Comments:** Results Page 12. You write: The radius of gytation of nanoliposome was formed.... Please clarigy because the Rg was not formed, the nanoliposopme was formed and the Rg allow you to measure the nanoliposome formation. I suggest you to be more descriptive about your MD simulation. **Responses:** Buat more explanation of each figures and the conclusion. ==> DONE
16	**Comments:** Results Figure 4. Why did you put a couple of figures per section. It would be better if you use boxes to separate these figures. **Responses:** We have revised the figure. We clarified this figure by providing box-separating figure for each properties.
17	**Comments:** Results Page. 16, The phrase "structures were generated in the molecular 332 modeling simulation without the electrostatic energy", how did you omit the electrostatic energy?? Please clarify It seems that the basis of molecular dynamic simulation are not clear, such as potential energy and the components of the same. Please review this concepts in order to improve the discussion of your results. **Responses:** We apologize for incorrect word without – with.

Reviewer 2

No	Comments and Responses
1	**Comments:** Page 1. Title: The author uses the word "Nanoliposom" instead of "Nanoliposome". Please change. **Responses:** Thank you very much for the comments given. We have revised the manuscript as follows:
2	**Comments:** Page 2 line 70: The information can be expanded by mentioning physicochemical parameters as size and zeta potential. They are important for the stability. **Responses:** The information of empirical particle size was mentioned. On the other hand, we are not providing zeta potential information since this study working on evaluating empirical results with the molecular modelling without any other surfactant both ionic and non-ionic. We are focused on generating liposome model rather than liposome stability.
3	**Comments:** Page 4 line 94: The authors didn´t mention the results found by the aforementioned researchers. **Responses:** Siwko et al. (2009) was stated that the presence of resorcinol may increase stability of lipid bilayer membrane. Hence, it was studied in our research the effect of 4-*n*-butylresorcinol, a resorcinol derivative, toward membrane stabilization.
4	**Comments:** Methodology: the authors must clarify if the liposome size reported is the radius or the diameter. It is desirable that they clearly explain how the liposome size is calculated from the radius of gyration. **Responses:** In this research, there were two approaches to be evaluated both in silico and empirical result of nanoliposome formulation. In the in silico study, we evaluated the radius of gyration to observe the size of formed liposome, while in the empirical observation the diameter of the molecules were evaluated. These parameters were comparable since the radius of gyration data were converted and calculated five times as stated in method section.
5	**Comments:** Page 5 line 151: Concentration of 4-n- butylresorcinol was not given. **Responses:** Tinggal nambahin konsentrasi. MD pakai jumlah molekul 2400 (123 mol), 2800 (143 mol), 3200 (164). Di empiris : 0.1%. Done.
6	**Comments:** Page 6 line 172: the authors say that "The temperature in the formulation was selected at 500 °C ..." Please correct.

	Responses: This section was revised. The correct one is 50°C.
7	**Comments:** Page 7 line 200: The angle and the temperature used in the liposome size measurements are not given. It should be clarified if the size distribution was measured by intensity, number or volume, since choosing one of these parameters over the others give variations in size. **Responses:** This section was revised as follow: "The determination and distribution process in the system was conducted using particle size analyzer instrument, Horiba SZ-100, based on the scattering dynamic light principle with temperature of 25.0°C and angle of 90°.
8	**Comments:** Figure 1 y 3: The authors didn´t explain why there are 2 blue lines and 2 two green lines. Please explain what each means. The chosen colors are difficult to distinguish. **Responses:** We have revised the figure and its caption.
9	**Comments:** Figure 4: Why they put two images for each system? Please clarify. **Responses:** We have revised the figure. We clarified this figure by providing box-separating figure for each properties. Dah dijawab di Reviewer 1 comment 16.
10	**Comments:** Table 6: Please add the liposome sizes found for all systems studied by MD. (2400 an 2800 are missing). **Responses:** Yang tidak optimal tidak diuji secara empiris, namun kita sarankan for future study sebagai control negative.
11	**Comments:** Figure 6: Specify the size of the scale bar in the caption of figure 6, since it is not visible in the photo. The authors should mention the diameter measurement of the liposomes shown in figure 6 by TEM. **Responses:** Diedit diperjelas keterangan skala.
12	**Comments:** The authors should include the liposome size distribution graphs obtained from the DLS, to see if there is a single population or not. **Responses:** Tinggal namabahin kurva distribusi, nambah Figure.==> DONE note tolong dijelaskan kalau yang kita lakukan empiris adalah liposom dengan zat aktif ya.. hasil sudah saya edit di dalam naskah
13	**Comments:** Do the number of drug molecules affects the liposome size?. Please clarify.

	Responses: Di pemodelan ada data mengenai pengaruh jumlah mol obat terhadap ukuran liposome, namun, at the moment belum bisa menguji variasi jumlah mol obat di uji empiris, disarankan for the future study.
14	**Comments:** It is desirable that the authors perform the experiments with the 3200 system without drug in order to corroborate their MD results. Also mention how reproducible is their experimental procedure, it seems that they only made once the experiments. **Responses:** Thank you for the suggestion. Tinggal nambahin data replikasi dan statistiknya.
15	**Comments:** Page 16 line 324: The authors say "It is also important to note that the slower liposome formation tends to give the phospholipid bilayer a chance to form a multiple connection, leading to an imperfect sphere or a bilayer bridge in the middle of the liposome." Please mention the corresponding figures. **Responses:** Sebutkan Figure 4 dan 5 . Penjelasan by Rini.
16	**Comments:** Page 17 line 374: The authors say "These insignificant results …." I guess the authors didn´t mean insignificant, please verify. I´m not sure what do the authors mean by "deal". Could you please clarify? **Responses:** Tinggal koreksi typo.

Reviewer 3

No	Comments and Responses
1	**Comments:** The idea of the work is good but 80% of the work is based on computational analysis it lacks experimental evidence so requires a few experimental data to validate it **Responses:** Thank you very much for the comments given. We have revised the manuscript as follows:

Outlook

Journal of Biomolecular Structure & Dynamics - Manuscript ID TBSD-2021-0257.R1 has been submitted online

From Journal of Biomolecular Structure & Dynamics <onbehalfof@manuscriptcentral.com>
Date Mon 4/5/2021 8:49 AM
To Rini Dwiastuti <rini_dwi@usd.ac.id>

05-Apr-2021

Dear Dr Dwiastuti:

Your manuscript entitled "In Silico Modeling and Empirical Study of 4-n-Butylresorcinol Nanoliposome Formulation" has been successfully submitted online and is presently being given full consideration for publication in Journal of Biomolecular Structure & Dynamics.

Your manuscript ID is TBSD-2021-0257.R1.

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If you do not hear from the editor within 2 months, please contact me.

Thank you for submitting your manuscript to Journal of Biomolecular Structure & Dynamics.

Sincerely,
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Outlook

Journal of Biomolecular Structure & Dynamics - Decision on Manuscript ID TBSD-2021-0257.R1

From Journal of Biomolecular Structure & Dynamics <onbehalfof@manuscriptcentral.com>
Date Tue 5/11/2021 12:18 AM
To Rini Dwiastuti <rini_dwi@usd.ac.id>

10-May-2021

Dear Dr Dwiastuti:

Your manuscript entitled "In Silico Modeling and Empirical Study of 4-n-Butylresorcinol Nanoliposome Formulation", which you submitted to Journal of Biomolecular Structure & Dynamics, has been reviewed. The referee comments are included at the bottom of this letter.

The reviews are in general favourable and suggest that, subject to minor revisions, your paper could be suitable for publication. Please consider these suggestions, and I look forward to receiving your revision.

When you revise your manuscript please highlight the changes you make in the manuscript by using yellow highlight. Please also ensure that you submit a clean version using the file designation 'Manuscript with track changes removed.'

Please provide a reply to the referee comments; summarizing the changes you have made within the body of the manuscript in response to the referee comments, and any other response that you want the editor and the referees to note. You should submit it as a separate document along with manuscript files "Response to Decision Letter and Reviewer Comments". Upload this as the first document. You indicate in the space provided in the response box that a separate document has been uploaded.

The changes should be presented IN the revised paper, explaining the changes in the response document does not help the reader. The only manuscript that you upload should be the revised one with changes highlighted in yellow. Any other versions of the manuscript should not be uploaded.

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Once again, thank you for submitting your manuscript to Journal of Biomolecular Structure & Dynamics and I look forward to receiving your revision.

Sincerely,
Professor Sarma
Editor in Chief, Journal of Biomolecular Structure & Dynamics
rhs07@albany.edu

Referee(s)' Comments to Author:

Referee: 1

Comments to the Author
The authors of the Manuscript "In Silico Modeling and Empirical Study of 4-n Butylresorcinol Nanoliposome Formulation" have given relevant explanation for all the queries given and the proper correction for all the types of typographical errors have been made . Necessary references were also included. Thus the manuscript can be accepted in your esteemed journal for publication.

Referee: 2

Comments to the Author
Major corrections were made but there are still some issues that shoul be corrected.
Abstract: You say: "using molecular dynamics simulation followed by potential energy calculation to understand the dynamics of the liposome

assembly and the morphology". But the potential energy is not a parameter to study structural modifications, Rg give you the details that you mention above.

Please review the english and overall drafting of the manuscript.

5. conversion of soy lecithin frommolecular modelling: from where did you obtain the value: 100 nm?

the new reference does not have the same format than the original manuscript, please correct it.

Editor's Comments to Author:

Associate Editor
Comments to Author:
(There are no comments.)

In Silico Modeling and Empirical Study of 4-*n*-Butylresorcinol Nanoliposome Formulation

-- Response to the reviewers' comments –

Reviewer 1

No	Comments and Responses
1	**Comments:** The authors of the Manuscript "In Silico Modeling and Empirical Study of 4-n Butylresorcinol Nanoliposome Formulation" have given relevant explanation for all the queries given and the proper correction for all the types of typographical errors have been made. Necessary references were also included. Thus the manuscript can be accepted in your esteemed journal for publication. **Responses:** Thank you very much for the recommendation.

Reviewer 2

No	Comments and Responses
1	**Comments:** You say: "using molecular dynamics simulation followed by potential energy calculation to understand the dynamics of the liposome assembly and the morphology". But the potential energy is not a parameter to study structural modifications, Rg give you the details that you mention above. **Responses:** Thank you very for the suggestion. We have revised the statement as follow: The in silico modeling was performed using molecular dynamics simulation followed by radius of gyration observation to understand the dynamics of the liposome assembly and the morphology of the formed liposome (Line 29-32).
2	**Comments:** Please review the english and overall drafting of the manuscript. **Responses:** We have reviewed the overall draft of our manuscript and proofread the manuscript to the licensed language institution. The proofread certificate is attached.
3	**Comments:** conversion of soy lecithin from molecular modelling: from where did you obtain the value: 100 nm? **Responses:** We have revised the manuscript and added statements as follow: In this study, the expected empirical size of nanoliposomes was 100 nm, however, due to our limitation in facility (computer speed and power), the simulation of nanoliposomes formation with particle size of 100 nm was difficult to achieve in the *in silico* modeling. Hence, we converted the particle size into 20 nm which is equivalent to 0.077424 g/mL (7.74 g/100 mL) of soy lecithin where the simulation was able to run out. (Line 220-223).
4	**Comments:** the new reference does not have the same format than the original manuscript, please correct it.

	Responses: We have checked the references format and revised them accordingly. (Line xxx)

Outlook

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To Rini Dwiastuti <rini_dwi@usd.ac.id>

31-May-2021

Dear Dr Dwiastuti:

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In Silico Modeling and Empirical Study of 4-*n*-Butylresorcinol Nanoliposome Formulation

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Abstract

A study to incorporate *in silico* modeling with an empirical experiment has been carried out to formulate nanoliposome containing 4-*n*-butylresorcinol as the active ingredient. The *in silico* modeling was performed using molecular dynamics simulation followed by radius of gyration observation to understand the dynamics of the liposome assembly and the morphology

of the formed liposome. The empirical experiment was conducted by formulating the nanoliposome using soy lecithin phospholipid formula as suggested by the *in silico* modeling followed by determining its particle size as well as its shape. From their incorporation, it was found that phospholipid molecules with the number of 3200 were selected in formulating nanoliposome containing 4-*n*-butylresorcinol. The result of the nanoliposomes size observation in the modeling of 3200 lipid molecules was 87.01 (± 0.59) nm, whereas the size from the empirical study was 87.57 (± 0.06) nm.

Keywords: 4-*n*-butylresorcinol, formulation, *in silico* modeling, nanoliposome, simulation.

List of abbreviation: LUV: large unilamellar vesicles, SUV: small unilamellar vesicles, AFA: Adaptive Focused Acoustics™, CGMD: Coarse-Grained Molecular Dynamic, DMPC: dimyristoyl-phosphatidylcholine, DPPC: dipalmitoyl-phosphatidylcholine, TEM: Transmission Electron Microscopy, PIPC: palmitoyllinoleyl-phosphatidylcholine, vdW: van der Waals.

Introduction

Nanoparticle technology has become one of the trending interests in drug delivery system research in the last decade (Boisseau et al., 2011; Puri et al., 2010). Applied technology in nanoparticle formulation can be performed using two different methods, namely top-down and bottom-up methods (Singh et al., 2011; Adami et al., 2006). Drug delivery system improvement using lipid nanoparticle formulation has been developed by arranging phospholipid bilayer fragments as well as liposomes for a diverse administration route in the human body. Lipid nanoparticle formulation provided several advantages such as its low toxicity, which is proved by *in vivo* observation, and its capability to improve the physical stability of the active ingredient in the dosage form formulation (Akbarzadeh et al., 2013). Nanoparticle technology can be applied in the drug formulation with the encapsulation process by several matrices such as nanospheres, nanoliposomes, and nanoemulsions (Martien et al., 2012).

One of the lipid nanoparticle drug delivery systems that have been commonly developed is nanoliposome since it has been reported with several advantages such as increasing the efficacy and therapeutic index as well as improving the drug stability through the encapsulation system (Akbarzadeh et al., 2013). Liposome, a topical dosage form, has been developed in the drug formulation due to its good penetration into the skin (El Maghraby et al., 2001; El Maghraby et

al., 1999). Nanoliposome showed good activity of penetrating the cell wall and intercellular space due to their nano-size, i.e. < 200 nm (Adami et al., 2006). The trend of nanoliposome formulation development increased continuously since the formulation process was reported to play an essential role in drug encapsulation (Wang et al., 2011; Zhao et al., 2009). The physical stability of the dosage form could be maintained by increasing the drug encapsulation and reducing the liposome leakage during the storage (Eloy et al., 2014; Laouini et al., 2012).

Nevertheless, several formulation problems were possible to be found during the formulation of nanoliposomes such as time-consuming process, organic solvent selection consideration, and economical consideration for performing trial and error formulation. Computational methods have been used to predict drug delivery systems before drug formulations. It aims to minimize investment in drug design and development (Li, 2010). The modeling of liposome formation molecules with phospholipids 1,2- dipalmitoyl-sn-glycero-3-phosphatidylcholine had been carried out by Koshiyama and Wada (2016), showing that the formed liposomes have different shapes and sizes depending on the number of phospholipids being used. Study related to in silico modeling of liposome formation had also been carried out by Dwiastuti et al. (2016) using 1,2-dilauroyl-sn-glycero-3- phosphatidylethanolamine producing almost the same shape and size of the liposomes in empirical experiments. The difference between the two studies is the software being used in the in silico modeling simulation, however, it has not been accompanied by empirical verification of the liposome manufacture. All of these problems should be overcome by generating an accurate prediction considering the atomic interaction between the drug and its delivery system using appropriate molecular modeling simulation (Hashemzadeh et al., 2020). Furthermore, the number of water and lipid molecules obtained from the in silico modeling is correlated with empirical observations of the liposome formation. The conversion factor should be calculated to obtain a quantitative correlation of phospholipid numbers used both for simulation modeling and empirical observation (Jämbeck et al.,2014). The in silico modeling of liposome formation can be applied in a formulation study by employing mathematical prediction to achieve a good nanoliposomes drug delivery system supported by the computational approach (Dogra et al., 2019; Cern et al., 2017; Cern et al., 2014).

One of the liposome formulation methods is a lipid dispersion in an aqueous media with sonication along with controlling its temperature, which is the easiest method (Akbarzadeh,

2013). The previous study by Shen et al. (2015) had empirically approached the formulation of large unilamellar vesicles (LUV) or small unilamellar vesicles (SUV) liposomes using focused ultrasonic irradiation with the usage of Adaptive Focused Acoustics™ (AFA) (Shen et al., 2015). This applied method was performed at a low temperature without organic solvent and produced liposomes with a size of < 600 nm. One way to reduce trial and error in experimental study is using in silico modeling to predict sub-molecularly the formation of liposomes (Trisilowati and Mallet, 2012). The lipid dispersion in aqueous media with sonication energy and its controlled temperature can be used as the system in the modeling approach, which is the first step before starting the empirical study. Further, the in silico modeling study should be compared with the empirical observations to obtain a correlation between the number of phospholipid molecules resulted from the modeling with the number of phospholipid molecules in the empirical study.

In silico modeling in this study was described as a computational approach to study the interaction between drug molecules with the liposome phospholipid membrane and to present the whole molecular interaction during the liposome formation. Rissellada and Marink (2009) studied the effect of temperature and membrane composition during the simulation process towards structure and dynamic behavior of the liposome membrane with the diameter of 15-20 nm using Coarse-Grained Molecular Dynamic (CGMD) simulation (Risselada et al., 2009). Another study by Siwko et al. (Siwko et al., 2009), *in silico* modeling of dimyristoyl-phosphatidylcholine (DMPC) phospholipid used CGMD at 323 K to investigate the interaction between resorcinol and the bilayer lipid membrane. The result showed that the presence of resorcinol was interestingly increasing the membrane stability of the lipid bilayer systems. Hudiyanti et al. (Hudiyanti et al., 2014) also employed the CGMD for simulating the *in silico* modeling to prepare liposomes from various phospholipids. In this study, three types of liposome (origin, deformed liposome, and planar bilayer) were *in silico* simulated to interact with water, to give an insight understanding which type of liposome will give the most stable structure during 160 ns of the simulation time. Therefore, the use of CGMD in drug delivery system modeling has been commonly used to provide the best formula in the liposome production.

In this present study, 4-*n*-butylresorcinol was used as the drug instead of resorcinol to be applied as the drug model. It has activity as tyrosinase inhibitor for the management of pigmentation disorders, such as the topical treatment of hyperpigmentation (Kolbe et al., 2013). This resorcinol derivative is more stable than resorcinol due to the occurrence of the oxidation

process (Love et al., 2005). A double-blind study for melasma with 4-*n*-butylresorcinol application time of twice daily within 8 weeks was necessary to achieve the hypopigmenting effect, however, 12 adverse events including mild erythema, dryness, peeling, and desquamation have appeared (Khemis et al., 2007). The 4-*n*-butylresorcinol was encapsulated in liposomes to improve the stability and to reduce skin irritation through hydration of the epidermis (Huh et al., 2010; De Leeuw et al., 2009). The stability should be improved by encapsulating the compound into the complex of nanoliposome structure. Encapsulating phenol-related compounds such as 4-*n*-butylresorcinol aims to preserve the biological activity and to improve the stability of the active compounds, as well as to ensure controlled release of the latter. Encapsulation has the advantage of being a non-thermal stabilization approach, suitable for temperature-sensitive natural biologically active compounds. The wall material usually improves the stability of the active compounds by protecting them from direct exposure to air and light (Coradini et al., 2014; Love et al, 2005). The *in silico* modeling simulation in the initial step of the formulation was important to predict the physical properties of the lipid nanoparticle complex production. The technique of formulation was necessarily developed to produce nanoliposomes with the size of nano (<100 nm) in a short time usage of sonication. It was reported that the heating and sonication processes were important in the nanoliposomes formulation. Mozafari et al. reported that the production of nanoliposomes with the size of ±600 nm was successfully carried out by the heating process without any extrusion process in polycarbonate membrane as well as sonication (Mozafari et al., 2007). Another study reported that the particle size reduction (≤ 100 nm) occurred with an increase of sonication time for more than 21 minutes (Silva et al., 2010). Hence, it is important to consider the correlation between the *in silico* modeling approach and empirical observational results in an integrated research. This study aims to correlate molecular dynamic (MD) simulation and an empirical observation in the production of nanoliposomes using soy lecithin phospholipid and 4-*n*-butylresorcinol as the delivery system and active ingredient, respectively.

Materials and Methods

1. Materials

Soy lecithin phospholipid (Pharmaceutical Grade, Nacalai Tesque, Japan), redistilled water, 4-*n*-butylresorcinol (Pharmaceutical Grade, SHREEJI Pharma International, India), ethanol (Analytical Grade, Merck, USA).

2. Gas Chromatography-Mass Spectrometry Analysis (GC-MS) of Soy Lecithin

The GC-MS analysis was performed using a Shimadzu GCMS-QP2010S with Agilent HP-5ms column (30 m x 0.25 mm x 0.25 µm). The injector temperature was set to 310^{o}C. Helium was used as carrier gas at a constant flow rate of 0.40 mL/min. The column temperature was kept at 120^{o}C for 5 min and then increased from 120^{o} to 300^{o}C at 5^{o} C/min. As for the ion source, the temperature was set to 250^{o} C, and the interface temperature was set to 305^{o} C. The mass scanning was set from m/z 28 to 600.

3. Molecular Dynamics Simulation of Soy Lecithin

Coarse-grained molecular dynamics using MARTINI 2.0 force field were used to study the liposome formation of soy lecithin. Prior to the initial coordinate preparation, the coarse-grained structure of phospholipids (DPPC, POPC, and PIPC), cholesterol, and 4-*n*-butylresorcinol were prepared. The GC-MS analytical result was used to determine the composition of soy lecithin and to construct the initial coordinates according to the composition. The initial coordinate was prepared by a random placement of phospholipid and cholesterol in a 22 x 22 x 22 nm^3 box using the gmx insert-molecule module of GROMACS 2019.1 package on Centos 7.4. Three concentrations of lipids were used i.e., 2400, 2800, and 3200 lipids/boxes. Each concentration of lipid had two treatments, i.e., by adding 4-*n*-butylresorcinol and without 4-*n*-butylresorcinol (negative controls). The molecule numbers of 4-*n*-butylresorcinol being added were 123, 143, and 164 molecules for 2400, 2800, and 3200 lipids, respectively. In total, there are six different initial coordinates in a duplicate experiment. After the lipid was randomly placed, the box configuration was edited using a gmx edit conf module and solvated with 78.299 water beads using a gmx solvate module generating a system consisted of lipids and water with the size of 24 x 24 x 24 nm^3. The initial coordinates were then subjected to energy minimization using the gmx mdrun module with the steepest descent method. The resulting minimized system then simulated under NPT condition for 240 ns using 40 fs time step, 323 K temperature, and reaction-field

long-range electrostatic treatment. The temperature control was using the V-rescale method while the pressure control used the Berendsen barostat. The MD results were then processed with gmx energy module to generate the potential energy data and gmx gyrate module to generate the radius of gyration data. The data were then plotted using Grace. The trajectory file is visualized using VMD 1.9.3 to understand the dynamic of the liposome assembly and the morphology of the liposome.

4. Empirical Observations

The formulation of nanoliposomes with and without 4-*n*-butylresorcinol was carried out by dispersing an amount of soy lecithin over redistilled water using the heating method incombined with sonication. The formula was composed of soy lecithin and other ingredients, as presented in Table 1. The empirical observation was approached by formulating the nanoliposomes using the theoretical data of soy lecithin through *in silico* modeling of 3200 phospholipid molecules. The phospholipid being used in this study was soy lecithin having transition temperature within 50-60°C (De Leeuw et al., 2009). The temperature in the formulation was selected at 50°C alongside with the sonication for 30 minutes and 37 kHz of a bath sonicator. Soy lecithin was dispersed in 100 ml of redistilled water at 50°C until the nanoparticle system met the requirement of a polydispersity index (< 0.3). The selected temperature at 50° C being used during the formulation was performed to approach the temperature condition close to the *in silico* modeling parameters. The soy lecithin dispersed solution was then blended at a high speed. This aimed to minimize the bilayer fragment sheets that have been formed. The solution being formed was then homogenized using Ultraturac® for 1 minute at four scales and sonicated for a further 30 minutes (Dwiastuti et al., 2016; Dwiastuti et al., 2016) until the particle with <100 nm in size were observed. The product was then left to cool down at room temperature before a morphological as well as the particle size testing. After that, the product was stored in a refrigerator at 4-8°C.

Table 1. The nanoliposome formula on the empirical observation.

Materials	Amount
Soy lecithin	7.74 g
4-*n*-butylresorcinol	0.1% w/v
Redistilled water	100.00 mL

5. The conversion of soy lecithin from molecular modeling into empirical observation.

The soy lecithin being used in the empirical observation was based on the number of phospholipid molecules in nanoliposomes modeling. The concentration of phospholipid in the simulation of 3200 phospholipid molecules was 387.12 mg/mL, which equals to 0.38712 g/mL.

The amount of soy lecithin being used: 0.38712 g/mL x $\frac{\text{the box size in modeling}}{100\text{ nm}}$

$$: 0.38712\ \text{g/mL} \times \frac{20\text{nm}}{100\text{ nm}}$$

$$: 0.077424\ \text{g/mL} \sim 7.74\ \text{g/100 mL}$$

According to the conversion above, therefore, the soy lecithin being used here was 7.74 g in 100 mL of redistilled water.

In this study, the expected empirical size of nanoliposomes was 100 nm, however, due to our limitation in facility (computer speed and power), the simulation of nanoliposomes formation with particle size of 100 nm was difficult to achieve in the *in silico* modeling. Hence, we converted the particle size into 20 nm which is equivalent to 0.077424 g/mL (7.74 g/100 mL) of soy lecithin where the simulation was able to run out.

6. Determination of the particle size

The determination and distribution process in the system was conducted using particle size analyzer instrument, Horiba SZ-100, based on the scattering dynamic light principle with temperature of 25.0°C and angle of 90°. It was 0.50 µL of the sample transferred into a 25 mL of a volumetric flask, and then added by redistilled water up to the calibration sign. A volume of 2.00 mL of aliquot was transferred into cuvet for the measurement of 100,000 particles.

7. Determination of a lamellar particle

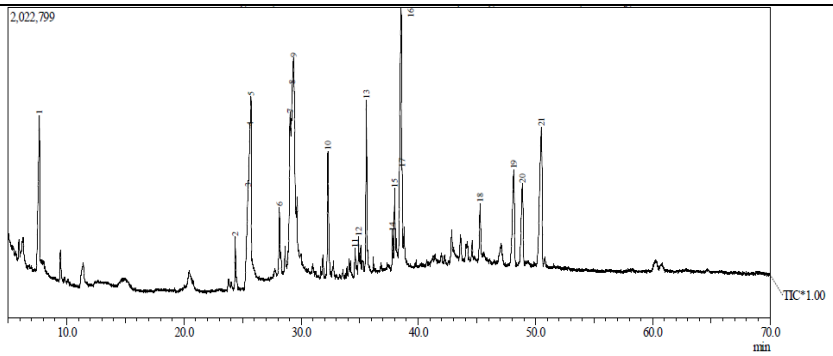
The observation of a lamellar particle used Transmission Electron Microscopy (TEM) JEM-1400 (Faculty of Natural Sciences, Gadjah Mada University, Indonesia). The determination of the lamellar particle was carried out to lipid nanoparticle with and without the presence of 4-*n*-butylresorcinol. A volume of 0.5 mL of the formula was added with 1 mL of redistilled water, and then dropped into an object plate. The electromagnetic transmission light was subjected to the plate, and then the particle morphology was observed at a suitable magnitude. Morphological results of the sample were displayed on the camera screen with no staining process. The result was then visualized as the lamellar particle shape.

Results and Discussion

The GC-MS of Soy Lecithin

We used the GC-MS, as this technique is suitable for lipid identification, in which the lipids are usually presenting in an ester form (Naviglio et al., 2017). Ester is usually volatile, therefore GC-MS is one of the suitable techniques to identify lipid both for its qualitative as well as quantitative prediction (Chiu et al., 2020). Furthermore, GC-MS is fast and cheap methods, providing the compounds library which could easily predict the identified peak in GC chromatogram which corresponds to the mass spectrum when it is available in the database. The % similarity in the MS result enhances the power on this instrument to be utilized in our lipid determination. The ion source is electron impact 70 MeV resulting to fragmentation of the parent molecule into its most stable peak (base peak) accordingly. However, this technique has disadvantage as the thermal stability of the compounds becomes the major issue (Koo et al., 2013). LC-MS could be the alternative method to reduce this thermal instability, unfortunately, we could not perform this method at the moment due to our limitation. The soy lecithin contents were analyzed using GC-MS method. The retention time, percentage of peak area, and the base peaks as well as their interpretation were furtherly detailed and presented in Table 2.

Table 2. GC-MS analysis result of the soy lecithin



No	Peak #	Retention Time (minute)	Area (%)	Base peak (MW)	Interpreted as (MW)
1	2	24.38	1.49	74.05	Methyl palmitate (270)
2	5	25.73	5.42	43.10	Palmitic acid (256)
3	6	28.15	1.50	55.05	Methyl oleate (296)
4	9	29.35	11.17	55.10	Linoleic acid (280)
5	13	35.58	5.25	43.05	Glycerol 1-palmitate (330)
6	16	38.52	14.09	55.10	2-hydroxy-1-(hydroxymethyl)ethyl ester linolein (354)
7	18	45.28	1.89	43.10	Cholesteryl myristate

8	19	48.13	4.85	43.05	5-ergostenol (400)
9	20	48.90	3.98	55.10	Stigmasterol (412)
10	21	50.49	10.09	43.05	Clionasterol (414)

Note: only he lipids and positive compound are shown

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In general, it was found that there were two primary compounds in soy lecithin namely fatty acid and sterol (Table 3). The detected fatty acids were recognized as palmitic acid, oleic acid, and linoleic acid; whereas, the sterol contents were recognized as clionasterol, 5-ergosterol, and stigmasterol.

Table 3. Soy lecithin primary component profiles

Component	Peak #	% Area	Total % Area
Fatty acid			
Palmitate	2, 5, 13	1.49 + 5.42 + 5.25	12.16
Oleate	6	1.50	1.50
Linoleate	9, 16	11.17 + 14.09	25.26
		Total fatty acids	38.92
Sterol			
5-ergostenol	19	4.85	4.85
Stigmasterol	20	3.98	3.98
Clionasterol	21	10.09	10.09
		Total sterol	18.92

From Table 3, it should be noted that only several lipids content was detected and calculated by the GC-MS method due to their thermal instability during analytical process. Therefore, the percentage of total area calculation was still below 100%. It can be assumed that palmitate content resulted in 54.32% since palmitate was found as major compounds in soy lecithin. Finally, the number of molecules for each lipid can be determined by multiplying the percentage and the total lipid, which can be seen in Table 4.

Table 4. Number of lipid molecules for each system in molecular modeling simulation

System #	Number of lipids	DPPC	POPC	PIPC	Cholesterol
1	2400	1304	36	606	454
2	2800	1521	42	707	530
3	3200	1738	48	808	606

Note. DPPC = dipalmitoyl-phosphatidylcholine; POPC = palmitoyloleyl-phosphatidylcholine; PIPC = palmitoyllinoleyl-phosphatidylcholine

The Potential Energy Calculation

The potential energy in the modeling of 2400, 2800, and 3200 phospholipid molecules were calculated. It can be noted that by increasing 4-*n*-butylresorcinol, the potential energy was found to be decreased (Table 5). The temperature adjustment was set to 50°C during the simulation due to the protocol published in the previous study by Siwko et al. (2009). Figure 1 depicted the graphical plot of potential energy-time dependent occurred during the simulation of 2400, 2800, and 3200 lipid molecules. The energy calculation involved in this study during the simulation processes were kinetic and potential energies.

Tabel 5. The comparison between the time, the potential energy, and the shape of liposome being formed during the simulation with the number of lipids and 4-*n*-butylresorcinol treatment variation.

Number of lipids	*n*-butyl resorcinol	T enclosed (ns)	E1 ($x10^6$ kJ/mol)	E2 ($x10^6$ kJ/mol)	E3 ($x10^6$ kJ/mol)	Shape
2400	No	90	-2.947	-2.962	-2.963	Rod
		19	-2.942	-2.948	-2.952	Spherical
	Yes	56	-2.954	-2.968	-2.969	Elliptical
		31	-2.947	-2.953	-2.957	Spherical
2800	No	40	-2.941	-2.949	-2.951	Spherical with torus-shaped cavity
		20	-2.948	-2.956	-2.959	Spherical
	Yes	68	-2.947	-2.957	-2.959	Elliptical with U-shaped cavity
		18	-2.950	-2.955	-2.958	Spherical
3200	No	12	-2.937	-2.939	-2.945	Spherical
		24	-2.939	-2.941	-2.945	Spherical with crescent-shaped cavity
	Yes	32	-2.945	-2.953	-2.955	Spherical with torus-shaped cavity
		14	-2.950	-2.954	-2.958	Spherical

T = The time until liposome fully enclosed
E1 = The energy at 10 ns
E2 = The energy when liposome fully enclosed
E3 = The energy at 240 ns

The potential energy involves in the liposome formation describes that without 4-*n*-butylresorcinol (blue lines) (Figure 1), it tends to have a potential energy higher than with 4-*n*-butylresorcinol (green lines). This indicates it is more stable system having 4-*n*-butylresorcinol than without this active ingredient, accordingly. Interestingly, this result deals with the study performed by Siwko et al., wherein the presence of resorcinol increased the liposome structural stability by joining the bilayer membrane, while inducing a proper arrangement of the phospholipid acyl chain leading to a lipid head group hydration.

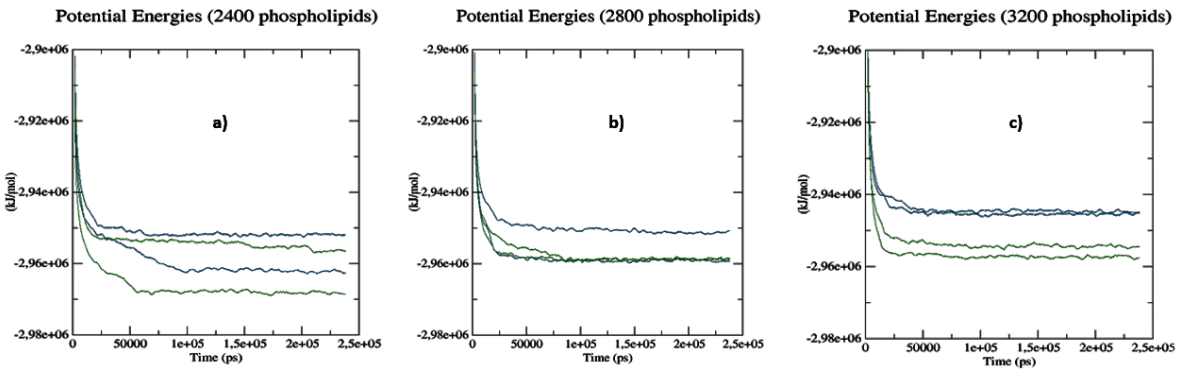

Figure 1. The graph plotting potential energy-time dependent occurred during the simulation of a) 2400, b) 2800, and c) 3200 lipid molecules. Note: Simulation with and without 4-*n*-butylresorcinol are shown by two green lines and two blue lines, respectively, as each treatment were performed in duplicate.

The potential energy contributes during the liposome formation is electrostatic and vdW energies, which are visualized in Figure 2. Figure 2a indicates that the electrostatic energy declines along with the increasing number of the phospholipid molecules as well as the 4-*n*-butylresorcinol, so does the vdW energy in Figure 2b. The total energy system in Figure 2d is the cumulative energy of kinetics (Figure 2c) and the potential energy of electrostatic (Figure 2a). From these graphs, the electrostatic and vdW are two of the most potential energies involved during the nanoliposome modeling. Although the electrostatic seems contributing the most stable energy with the system having the largest molecule number of phospholipid, however, the presence of 4-*n*-butylresorcinol is predicted to increase the system stability by inducing the phospholipid acyl chain to stabilize the forming bilayer membrane. Here, the vdW is also contributing into the system stability as this energy profile (Figure 2b) is identical with the electrostatic energy (Figure 2a). In conjunction, the total energy (Figure 2d) is in proportional with the potential energy as depicted in Figure 2a and 2b, whereas the kinetic energy does not have the same trend. This different trend could be due to the kinetic energy as being a fraction from the total energy system, so this could be neglected. In other words, the selected system is considered from the most stable energy profile, in which potential system employing electrostatic and vdW are the two best ones.

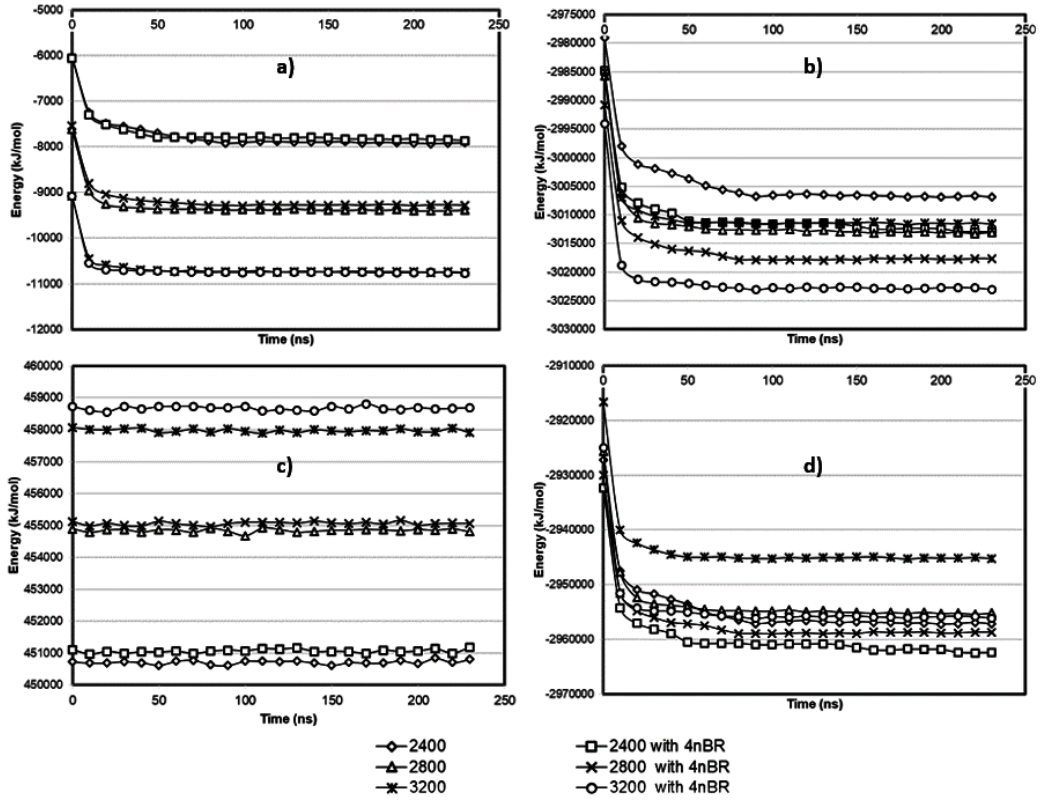

Figure 2. The graph plotting potential energy-time dependent occurred during the simulation of a) electrostatic, b) vdW, c) kinetic, and d) total energy.

The Radius of Gyration of *In Silico* Modeling Simulation

The radius of gyration can be described as a unit for the radius average from the molecule to the centre of mass to determine the liposome diameter. The radius of gyration allows to measure the nanoliposome formation during the simulation of 2400, 2800, and 3200 lipid molecules with and without the presence of *4-n*-butylresorcinol (Figure 3). The nanoliposomes morphological results of the simulation of 2400, 2800, and 3200 lipid molecules and their snapshots of nanoliposomes formation results during simulation can be seen in Figure 4 and Figure 5, respectively. The liposome radii being formed during the simulation are shown in Figure 3a-c with a duplicate simulation. The result indicates that the liposome radii increase along with the increasing number of both phospholipid and *4-n*-butylresorcinol molecules.

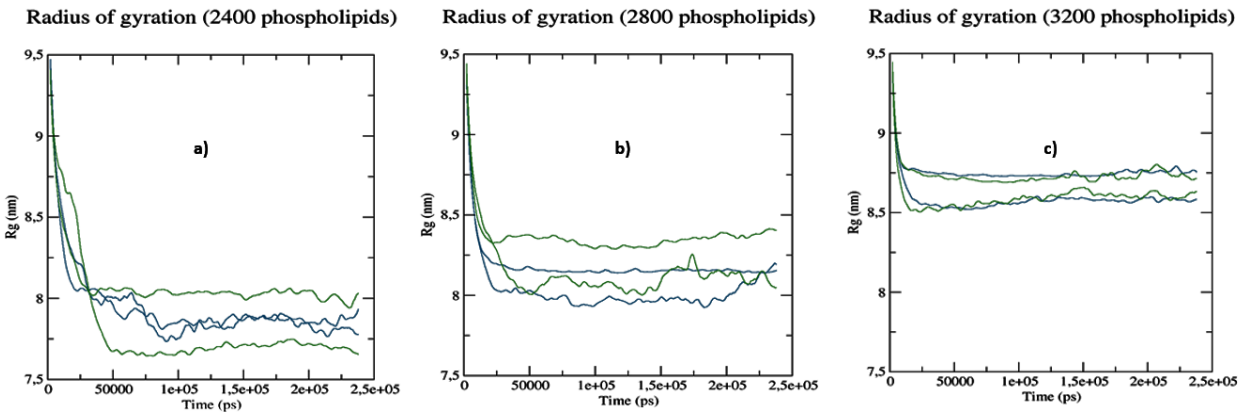

Figure 3. The gyration radius of the nanoliposomes being formed during the simulation of a) 2400, b) 2800, and c) 3200 of lipid molecules with (green) and without (blue) the presence of *4-n*-butylresorcinol.

The increasing liposome radii results in the increasing of the liposome size as its morphology is depicted in Figure 4. The shape of simulation having 2400 phospholipid molecules with *4-n*-butylresorcinol in the first replication more likely less spherical than the second one (Figure 4a). The similar result occurs in the system having 2800 of phospholipid molecules with *4-n*-butylresorcinol showing more less imperfect spherical shape. Interestingly, the system having 3200 phospholipid molecules with *4-n*-butylresorcinol shows the most spherical shape among others indicating its efficient atomic interactions between phospholipid and *4-n*-butylresorcinol. The inefficient atomic interactions could form the double connection between *4-n*-butylresorcinol and its corresponding phospholipid, and other phospholipid leading to the final imperfect spherical structure. From this simulation, system having 3200 phospholipid molecules is the most stable and reliable system predicted to produce liposome structure that will be used in the empirical study.

Properties	a	b	c
without *4-n*-butylresorcinol	Replication 1 Replication 2	Replication 1 Replication 2	Replication 1 Replication 2
with *4-n*-butylresorcinol	Replication 1 Replication 2	Replication 1 Replication 2	Replication 1 Replication 2

Figure 4. Nanoliposomes morphological results of the simulation of a) 2400, b) 2800, and c) 3200 lipid molecules with two replications for each lipid molecule number.

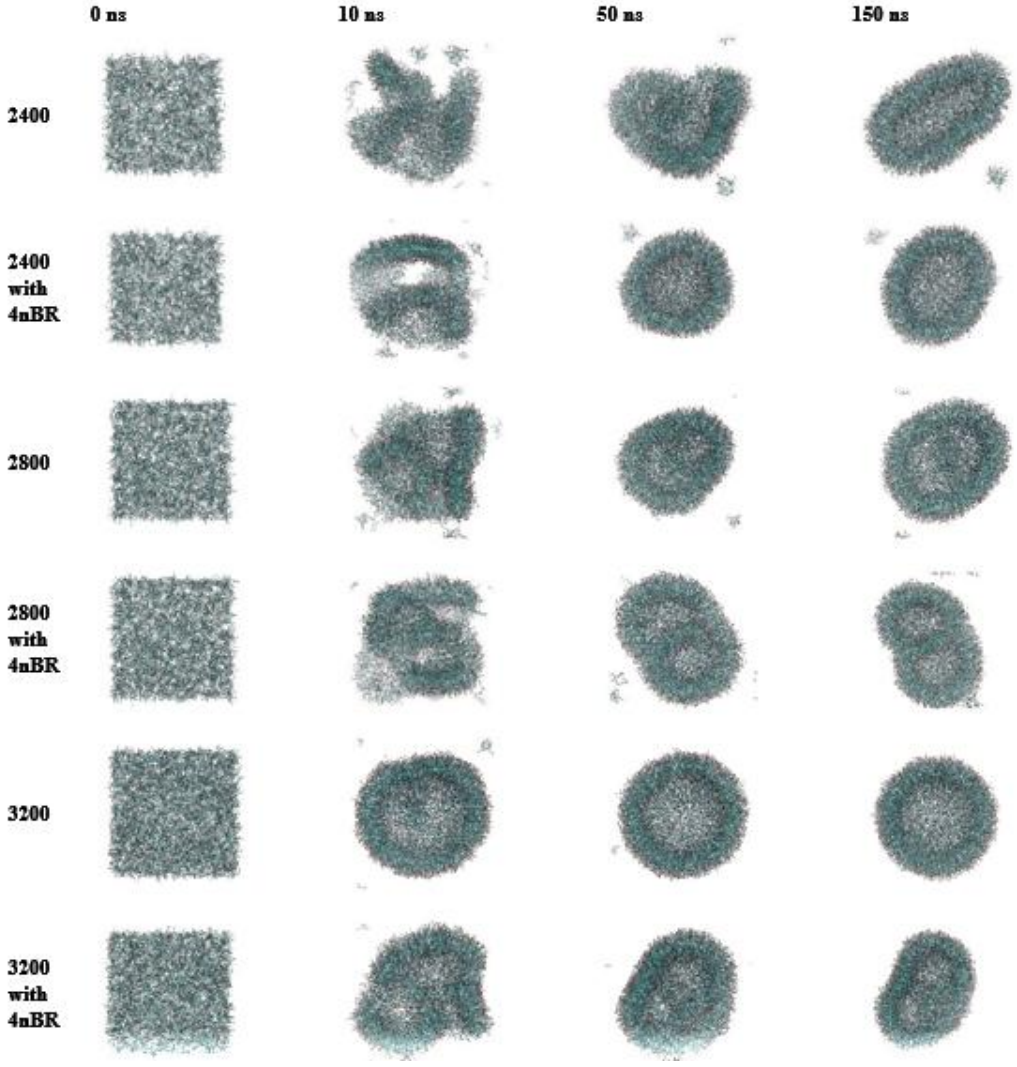

Figure 5. Snapshots of nanoliposomes formation results during the simulation

The Size of Nanoliposome with The Presence of 4-*n*-Butylresorcinol

The size of nanoliposome resulted from both molecular dynamics simulations and empirical observations were compared and presented in Table 6. In this presentation, the empirical liposome was prepared from the selected system only (3200 phospholipid molecules with *4-n*-butylresorcinol) because this system showed the most stable energy profile as well as its morphology. The distribution curve of the nanoliposome size can be seen in Figure 6 demonstrating the population of liposome particle size with three times replications which shows consecutive mode values i.e. 87.60, 87.50, and 87.60 nm with the standard deviation value is 0.06. The mode value is chosen representing the liposome size distribution because it represents the largest frequency of the liposome size, which is generated in the empirical study. The distribution curves exhibit a similar pattern so that it indicates a good repeatability in the particle size of the liposome.

Table 6. Results of nanoliposome size based on simulation and empirical observation for 4-n-butylresorcinol

Nanoliposome sizes based on the *in silico* simulations (3200 molecules)		**Nanoliposome sizes based on the empirical observation**		
Replication	**Nanoliposome sizes (nm)**	**Replication**	**Calculation mode (nm)**	**Polydispersity Index**
1	86.60	1	87.60	0.28
2	87.43	2	87.50	0.18
		3	87.60	0.24
Mean (±SD)	87.01 (± 0.59)	Mean (±SD)	87.57 (± 0.06)	

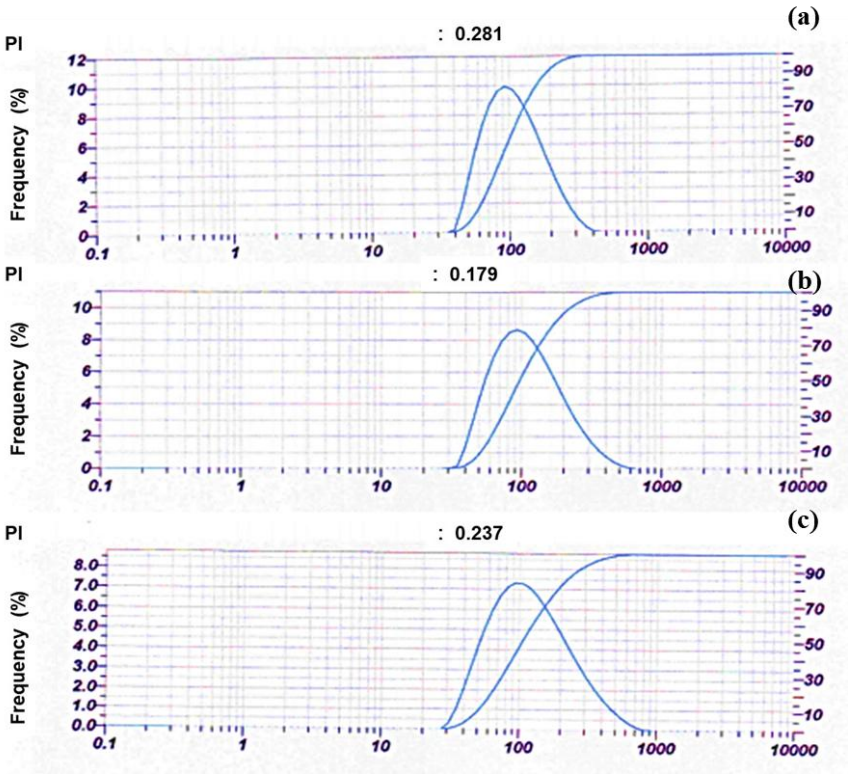

Figure 6. The nanoliposome size distribution curves in triplicate measurements exhibit a similar population with their consecutive mode values i.e. 87.60 (a), 87.50 (b), and 87.60 nm (c) having polydispersity index (PI) are 0.281, 0.179, and 0.237, respectively.

The Morphology of 3200 Phospholipid Molecules

The morphological observation of 3200 phospholipid molecules from the simulation model was compared to the empirical observation without the presence of 4-*n*-butylresorcinol, which can be seen in Figure 7, while the interaction between drug and the membrane model was depicted in Figure 8.

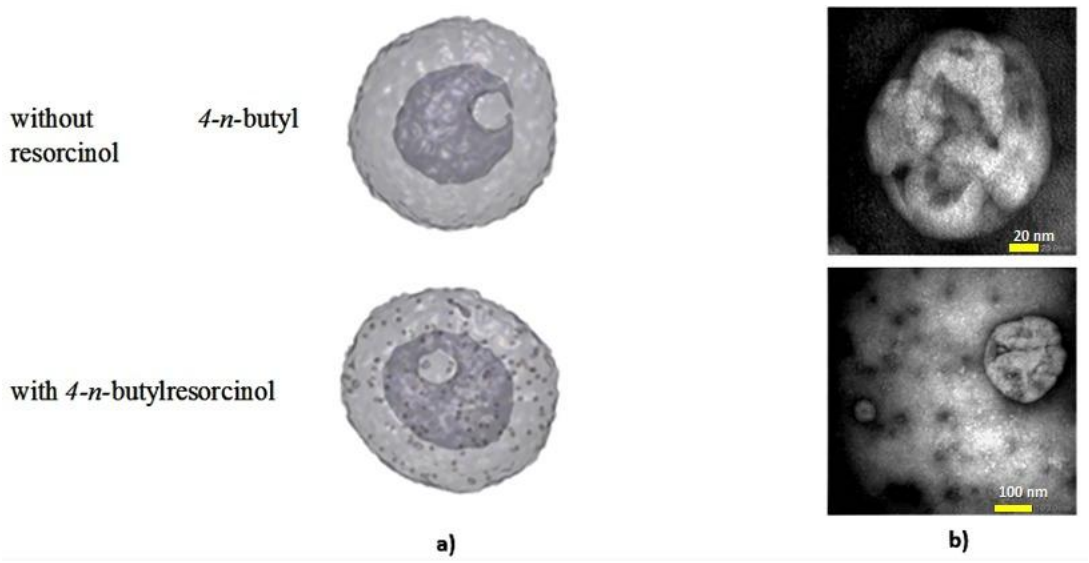

Figure 7. The nanoliposome morphology being formed from a) the modeling and b) the empirical observation on 3200 lipid molecules.

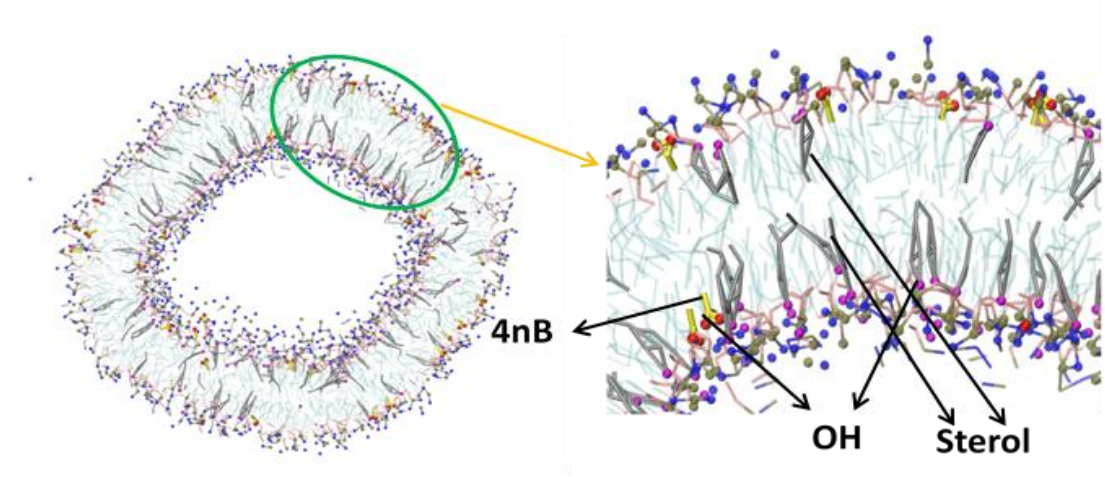

Figure 8. The interaction of the model compound in the lipid membrane

The GC-MS method was utilized to determine the chemical composition of the soy lecithin as listed in Table 2. The fatty acids of the phospholipid and sterol were used as the platform in the nanoliposomes modeling. The identified compositions of fatty acid from the GC-MS were palmitic acid, oleic acid, and linoleic acid. It is appropriate with the composition of soy lecithin theoretically (Perkins et al., 1995; Van Hoogevest et al., 2013). The sterol being composed in the soy lecithin was 5-ergosterol, stigmasterol, and clionasterol (Table 3). The percentage of fatty acid and sterol as calculated in Table 3 was used as the basic calculation to decide the composition of the nanoliposomes modeling.

The nanoliposomes modeling with and without the presence of 4-*n*-butylresorcinol used three variations of the lipid molecule number, i.e. 2400, 2800, and 3200 molecules. This observation was aimed to predict the possibility of the nanoliposomes formation with and without the presence of 4-*n*-butylresorcinol by modeling on a few lipid molecules being used. The decision of selecting the number of lipid molecules was applied and referred to the study by Koshiyama and Wada (2016). The results indicated a formation of nanoliposomes from modeling using 2400, 2800 and 3200 molecules inside 22 nm^3 of the box size (Figure 2).

According to the graphical energy presented in Figure 1 and 2, in general, it was showed that the potential energies were stronger for a lower lipid number. This trend appears as the lower number of lipids, the higher number of waters, which contributes to the stronger electrostatic and vdW interactions. Whilst in term of the velocity and its stability, both the energy graph as well as the radius of gyration graph, shows that at a higher number of lipids, the liposomes were formed faster and quickly stabilized. It is also important to note that the slower liposome formation tends to give the phospholipid bilayer a chance to form a multiple connection, leading to an imperfect sphere or a bilayer bridge in the middle of the liposome (see Figure 4b). Therefore, the 3200 lipids molecule was subjected to the empirical nanoliposomes formulation by with and without the presence of 4-*n*-butylresorcinol. The formation of nanoliposomes in the molecular modeling involves kinetic and potential energies in the form of electrostatic and vdW interactions. According to the energy calculation results in the modeling simulation at 50°C, the nanoliposome system was stable at about 100 ns (Figure 1 and Figure 2). Nanoliposome structures were generated in the modeling simulation with the electrostatic energy. It may be resulted from the interaction between electronegative atoms in the phosphate

group of the phospholipid with the hydrogen atom from the water molecules around the system. Furthermore, an electrostatic interaction between polar groups of phospholipid during simulation also occurs. The lowering of vdW energy indicated a similar graphical profile with the lowering of electrostatic and total energies in the system. The interaction between nonpolar group during the simulation resulted vdW energy. With the longer simulation time, the electrostatic, vdW, and the total energy were decreased due to the system stabilization in order to achieve the nanoliposomes structure (Figure 5) for simulation, with and without *4-n*-butylresorcinol in three different molecules number (2400, 2800, and 3200). The system stabilization has been achieved when the unilamellar nanoliposomes was formed in 100 ns. Moreover, the electrostatic, vdW, and total energies were showed to be decreased in the both system with and without *4-n*-butylresorcinol during the simulation. The vdW energy depicted interactions between nonpolar groups of phospholipid molecules and nonpolar group of *4-n*-butylresorcinol. Chemical properties of the nonpolar model compound might affect the vdW interaction at the initial and at the end of the simulation. The interaction between 4-*n*-butylresorcinol-lipid and lipid-membrane during the simulation process resulted similar characteristic results with the previous study (Siwko et al., 2009), where the hydroxyl (-OH) group of *4-n*-butylresorcinol interacted with the hydrophilic group of the phospholipid compound (Figure 7). The presence of *4-n*-butylresorcinol increased the vdW interaction between non polar groups, whereas the electrostatic interaction in phosphate group with hydrogen atom from water was decreased since there was several hydrogen bonding between two phospholipid molecules. The morphological results of the simulation (Figure 5) indicated that nanoliposomes was formed into unilamellar structure. The simulation with 3200 phospholipid molecules resulted a similar form of nanoliposomes compared to the empirical observation result (Figure 6).

The conversion of the phospholipid from the simulation was done using a concentration in that 3200 molecules by 0.38712 g/mL with the conversion factor of 0.2. This factor was a ratio between the box size used in the simulation (20 nm) and the actual box should be used in the empirical observation, i.e. 100 nm. The parameter being observed in the nanoliposomes product was the size and the morphology of the nanoliposomes being formed. The result of the nanoliposomes size in the molecular modeling of 3200 lipid molecules was 87.01 nm (± 0.59), whereas the size from the empirical study was 87.57 (± 0.06). These results indicated that there were no significant different for the size and the morphology of the nanoliposomes formed with and without the presence of 4-*n*-butylresorcinol, which found a deal between these *in silico* and empirical studies. However, this study has some limitations regarding with no physicochemical parameter evaluation and empirical studies for 2400 and 2800 phospholipid molecules, which prompts us to do these experiments in our future study.

Conclusions

Molecular dynamics simulation was proved as the tool that is strongly predicting the dynamics behavior of 4-*n*-butylresorcinol upon nanoliposome formulation. The program was able to predict the number of phospholipid molecule during the formulation which resulted in agreement between *in silico* and empirical study. It was found that phospholipid molecules with the number of 3200 were selected in formulation process of nanoliposome containing 4-*n*-butylresorcinol. The modeling and empirical studies resulted the molecules size of 87.01 (± 0.59) and 87.57 (± 0.06) nm, respectively. These significant results indicated the deal between the *in silico* modeling and the empirical observation in formulating nanoliposome.

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Disclosure statement

No potential conflict of interest reported by the authors.

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