Dynamics insights into aggregation of phospholipid species with cholesterol and vitamin C

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Abstract
This paper provides dynamic insight into the aggregation profile of systems containing six different phospholipid species, cholesterol, and vitamin C thru Coarse-Grain Molecular Dynamics (CGMD) simulations. The simulation used 42 systems, and each system was composed of 220 molecules of each phospholipid species, a varied number of cholesterol molecules (0, 11, 22, 33, 66, 88), and 10 vitamin C molecules. The phospholipid species were DLPE, DOPE, DLiPE, DOPS, DLiPS, and DLiPC. We found curved bilayer, toroidal bilayer, concave micelle, disc-like bilayer, planar bilayer, and liposome structures in the systems during the 40 ns simulation. The systems with a ratio cholesterol:phospholipid between 15% and 40% formed liposomes regardless of the phospholipid species. Cholesterol is positioned in the liposome bilayer while vitamin C is encapsulated in the aqueous core of liposomes for all cholesterol compositions. The cholesterol influences the liposome formation of various phospholipid species and the encapsulation of vitamin C in the liposome structure.

Keywords
Coarse-Grain Molecular Dynamics, liposome, membrane, self-assembly, vesicle

Introduction
Over the past few years, the use of vitamin C as an active agent has increased rapidly. People consider that vitamin C has antioxidant and other beneficial properties such as whitening effect. In addition, vitamin C boosts the body's immune system by speeding up the production of T cells and B cells that play a role in killing bacteria and viruses, also aiding other cell types in the immune system (Alkandahri et al. 2018). Despite the above properties, vitamin C is unstable to light, temperature, and changes in pH (Sheraz et al. 2015). Due to these shortages, we need an effective delivery method for vitamin C.

Phospholipid encapsulation is the right choice because they are more affordable and easy to obtain than other delivery methods. Phospholipids have the self-assembly ability to form structures such as liposomes. Research showed that liposomes encapsulate vitamin C and that cholesterol addition can overcome leakage of liposomes (Hudiyanti et al. 2018, 2019a; Liu et al. 2020). However, molecular
dynamics information on structural characteristics and the aggregation profile of phospholipid self-assembled structure with cholesterol and vitamin C are not established yet.

Coarse-Grained Molecular Dynamics (CGMD) simulation suits an exemplary method (Joshi and Deshmukh 2020; Liwo et al. 2021). It provides information about the molecular dynamics of the systems that we may not obtain experimentally without using a high-specification computer. The CGMD simplifies one to four heavy atoms into one bead while still carrying the properties and dynamics of those molecules. It also allows modification of the simulated environment and presents similar data as standard computer software. Previously, molecular dynamics simulation with coarse-grained modeling has determined the aggregation profile of self-assembled phospholipid structure and proved that the liposomes are metastable structures and water molecules are within the liposome core (Hudiyanti et al. 2014). However, the molecular dynamics of phospholipids with cholesterol and vitamin C in their self-assembled structures have not been fully established. Therefore, this study provides a new understanding of the liposomes’ structural characteristics and aggregation profiles that contain phospholipids, cholesterol, and vitamin C.

**Materials and methods**

The research consisted of three main stages, namely (i) the system preparation consisting of phospholipid molecules, cholesterol, and vitamin C, (ii) the molecular dynamics simulations, and (iii) the analysis of phospholipid aggregation profile data. Molecular models were derived from Protein Data Bank, and PubChem (Wang et al. 2009), then prepared with the Open Babel package (O’Boyle et al. 2011) and modified with Molefacture on Visual Molecular Dynamics (VMD) package (Humphrey et al. 1996). Fig. 1 presents all the phospholipids, cholesterol, and vitamin C structures used in the simulation.

The coarse-grained structure of phospholipids follows Hudiyanti (Hudiyanti et al. 2014), cholesterol coarse-grained structure follows the “angle-corrected” model (CT3-Me2b) of the Daily (Daily et al. 2014). The coarse-grained structure of vitamin C modifies the model of HSP of Periole & Marrink (Periole and Marrink 2013). Ten to 14 beads represented each phospholipid molecule, ten beads modeled cholesterol molecules, and four beads modeled vitamin C molecules. Fig. 2 presents the coarse-grained structure of all molecules used in this simulation. The systems composition contained 220 phospholi-

![Figure 1. Molecular structure of Phospholipids (a–f), cholesterol (h), and vitamin C (i). a 1,2-Dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE) b 1,2-Dilauroyl-sn-glycero-3-phosphoserine (DLPS) c 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) d 1,2-Dioleoyl-sn-glycero-3-phosphosperine (DOPS) e 1,2-Dilinoleoyl-sn-glycero-3-phosphoethanolamine (DLePE) f 1,2-Dilinoleoyl-sn-glycero-3-phosphoserine (DLePS) h Cholesterol i Vitamin C.](image-url)
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Phospholipid molecules (Table 1), cholesterol (0; 11; 22; 33; 66; 88 molecules), and ten vitamin C molecules. Molecules were randomly placed in an 11 nm cube-shaped box using the Packmol package (Martínez et al. 2009).

**Table 1.** Phospholipid species used in this study.

| Phospholipid species | Number C atoms: Number of Double Bonds of Phospholipid acyl groups |
|----------------------|---------------------------------------------------------------|
| Ethanolamine Head Group (PE) | 12:0 (DL) | 18:1 (DO) | 18:2 (D Li) |
| Serine Head Group (PS) | DLPS | DOPS | DLiPS |
| Choline Head Group (PC) | – | – | DLiPC |

Coarse-grained-based residues are applied and identified on the system with AutoPSF based on MARTINI Force Field (Periole and Marrink 2013) using the VMD package. VMD package is used to convert all-atom structures into coarse-grained structures. We applied a solvation process to the system to provide an aqueous environment. Meanwhile, ionization is designed to neutralize the system by adding Na⁺ and Cl⁻ ions. Based on the MARTINI Force Field, each bead interacts with the potential pair of Lennard-Jones (LJ) and van der Waals forces. As for modeling electrostatic interactions between amino head groups on the system based on Coulomb’s (Marrink et al. 2007). The total energy system was initially minimized and simulated in an aqueous milieu with a density of 0.00609 atoms/Å³ using the Scalable Molecular Dynamics (NAMD) package to determine the self-assembled phospholipid structure. We performed the simulation process in the NPT ensemble (conditioning pressure state constant around 1.01325 bar). The minimization was applied to reach the system’s lowest energy state by minimizing interaction between atoms, adjusting the structure to the force field, providing homogenous solvent distribution, and reducing steric collisions in the system (Hudiyanti et al. 2014). This lowest energy indicated the system’s most stable conformation before aggregation proceeded. The achievement of energy convergence during minimization in 0.6 ns shows the lowest

Figure 2. The coarse-grained structure of all molecules in the simulation. The phospholipid in fig (a–g): light blue bead for ethanolamine head group, purple for serine head group, orange for choline head group, light brown for phosphate, pink for glycerol backbone, dark brown for hydrocarbon tail group, and green for the double bond on hydrocarbon tail group; dark blue beads for cholesterol in fig (h); and yellow beads for vitamin c in fig (i). a 1,2-Dilauroyl-1-sn-glycero-3-phosphoethanolamine (DLPE) b 1,2-Dilauroyl-1-sn-glycero-3-phosphoserine (DLPS) c 1,2-Diacyl-1-sn-glycero-3-phosphoethanolamine (DOPE) d 1,2-Diacyl-1-sn-glycero-3-phosphoserine (DOPS) e 1,2-Diacyl-1-sn-glycero-3-phosphoethanolamine (DLiPE) f 1,2-Diacyl-1-sn-glycero-3-phosphoserine (DLiPS) h Cholesterol i Vitamin C.
total energy system to perform dynamics simulations. The dynamics simulation duration is 40 ns. We used periodic boundary conditions (PBC) for the simulations and 40 fs time step integration. The VMD package visualizes and analyzes both the preparation and simulation outputs. The simulation gives the aggregation profile of the self-assembled structures. We analyzed important parameters: Root Mean Square Deviation (RMSD), aggregate structure, the total energy of the system, liposomes lifetime, area per lipid, membrane thickness, and liposomes size.

Results and discussion

The molecular dynamics simulation provides an overview of the self-assembled process and properties of phospholipids with cholesterol and vitamin C. Table 2 presents aggregate structures and total energy related to the systems. Parameters related to liposome structures during 40 ns of simulation, namely liposome lifetime, area per lipid, membrane thickness, and liposome size, were presented in Table 3. The phospholipid species, the cholesterol concentration in the system, and the addition of vitamin C affect the aggregate structures and parameters. Particular attention was directed to the systems that formed liposomes during simulations. Based on previous research conducted by Hudiyanti (Hudiyanti et al. 2014), the structure of liposome as a self-assembled phospholipid structure can be obtained in about 40 ns simulation time for as many as 256 phospholipid molecules. Therefore, in this research, the simulation time of 40 ns is assumed to be sufficient to simulate 220 phospholipid molecules until liposomes are formed.

RMSD analysis

RMSD value describes the average distance between each atom in the system at a specific time. A molecule with an RMSD value that does not change much over time indicates a stable molecular conformation. The structural stability of the systems during simulation is suggested by the converging curve of RMSD, for example, of system 220DLiPE-88Chol-10VitC, as shown in Fig. 3. Convergency of RMSD value during simulation becomes the basis for further parameter analysis. All data presented are collected and calculated after conforming to the convergency of the RMSD value between 4.21–4.83 Å during 40 ns simulation time.

Aggregate structures

We initiated the simulation with a random molecular position. After some time, the scattered molecules will interact and form various self-assembled structures such as toroidal bilayer, disc-like bilayer, liposomes, and planar bilayer, as presented in Fig. 4. The formation of liposomes can be as early as 3.48 ns for DOPS and as late as 36.48 ns for DLPS after the simulation commence, see Table 3. In Fig. 4 a system formed liposomes, and vitamin C with its hydrophilic property was encapsulated in the aqueous compartment or the liposomes core together with water molecules. Besides that, unencapsulated vitamin C molecules are also found outside the liposomes and reside among water molecules of the system.

Further from Table 2, we saw that the aggregate structures and the total energy did not significantly change when introducing vitamin C to the systems. These phenomena suggested that the addition of vitamin C did not influence the formation of liposomes in all scenarios. Meanwhile, cholesterol with the hydrophobic
property was encapsulated in the liposome bilayer membrane. Other systems formed self-assembled structures other than liposomes (Fig. 4b–e). The system compositions influence the formation of the liposomes. A certain amount of cholesterol is required to form liposomes that will encapsulate the vitamin C. Small amount of cholesterol, i.e., 0, 11, 22 molecules, for most systems were inadequate to induce the formation of the liposomes. Contrarily systems with high amounts of cholesterol, i.e., 33, 66, and 88 molecules, are more capable of forming liposomes and at the same time encapsulating vitamin C. Besides increasing the possibility of forming liposomes, an increase in cholesterol also impacts the stability of liposomes, as indicated by the increasing lifetime of liposomes, as listed in Table 3. Cholesterol inhibits the deformation of the membrane, causing the liposomes to stay intact until the end of the simulation.

### Total energy system

Structural changes that occur during the aggregation process of forming a self-assembled structure were accompanied by a decrease in the system's total energy, as seen in Fig. 5. The total energy decreases until it reaches minimum energy when the simulation ends and forms a converging curve indicating the stability of the system's final structure. The total energy of liposome formation was the highest amongst other self-assembled structures that is due to the liposomes bilayer's high curvature. Liposomes formation occurred at total energy from -62,939 to -55,778 kcal/mol. In general, the bilayer structures had lower total energy compared to liposomes. The higher the amount of cholesterol, the higher is the total energy of the system. Amongst all phospholipid species with different head groups, the total energy of the system composed of PC was higher than PE and PS, as seen from the system total energy data in Table 2. The system composed of phospholipids with 18 carbon atoms in their acyl chains has higher total energy than the 12 carbon atoms. Meanwhile, the system composed of phospholipids with one double bond has higher total energy than two double bonds. The order of the total energy based on the nature of the acyl chains in each phospholipid species from the lowest was DLP(12:0)<DLP(12:1)<DLP(18:2)<DOP(18:1)<DLPE(18:2)<DOPE(18:1)<DLP(18:2).

### Liposomes size, membrane thickness, and area per lipid

Table 3 showed that cholesterol affected the liposomes size, membranes thickness, and area per lipid of the liposome membrane of each system. Increasing the amount of cholesterol in the system increases the liposome's size and the

| Phospholipid Species | Liposome Composition | Total energy (kcal/mol) | Occurrence Time \( t_{\text{occurrence}} \) (ns) | Lifetime (ns) | Area per lipid (nm²/lipid) | Membrane thickness (nm) | Liposome size (nm) |
|----------------------|----------------------|-------------------------|---------------------------------------------|---------------|----------------------------|-----------------------|------------------|
| DLPE                | 220-33-10            | -62,138                 | 31.76 - 40.00                              | 8.27          | 1.20                       | 4.42                  | 8.00             |
|                      | 220-66-10            | -61,142                 | 26.80 - 40.00                              | 13.20         | 1.07                       | 4.36                  | 8.30             |
|                      | 220-88-10            | -58,692                 | 18.08 - 40.00                              | 21.92         | 0.98                       | 4.78                  | 8.40             |
| DLPS                | 220-33-10            | -62,939                 | 36.48 - 40.00                              | 3.52          | 1.15                       | 4.72                  | 8.50             |
|                      | 220-66-10            | -61,339                 | 34.84 - 40.00                              | 5.16          | 1.03                       | 4.96                  | 9.00             |
|                      | 220-88-10            | -59,412                 | 13.76 - 40.00                              | 26.24         | 0.95                       | 4.85                  | 8.50             |
| DOPE                | 220-22-10            | -59,311                 | 30.00 - 40.00                              | 10.00         | 1.23                       | 4.97                  | 8.50             |
|                      | 220-66-10            | -56,922                 | 28.80 - 40.00                              | 11.20         | 1.05                       | 4.99                  | 9.00             |
|                      | 220-88-10            | -56,014                 | 27.08 - 40.00                              | 12.92         | 0.98                       | 5.20                  | 9.20             |
| DOPS                | 220-66-10            | -57,997                 | 6.36 - 40.00                               | 33.64         | 1.01                       | 5.29                  | 9.00             |
|                      | 220-88-10            | -56,534                 | 3.48 - 40.00                               | 36.52         | 0.94                       | 5.43                  | 9.50             |
| DLiPE               | 220-22-10            | -59,596                 | 29.44 - 40.00                              | 10.56         | 1.23                       | 4.88                  | 8.72             |
|                      | 220-33-10            | -58,985                 | 28.68 - 40.00                              | 11.32         | 1.18                       | 5.12                  | 8.72             |
|                      | 220-66-10            | -57,440                 | 29.68 - 40.00                              | 10.32         | 1.05                       | 5.07                  | 8.90             |
|                      | 220-88-10            | -56,200                 | 21.80 - 40.00                              | 18.20         | 0.98                       | 5.06                  | 9.00             |
| DLiPS               | 220-66-10            | -58,627                 | 23.36 - 40.00                              | 16.64         | 1.02                       | 5.07                  | 9.00             |
|                      | 220-88-10            | -57,111                 | 20.32 - 40.00                              | 19.68         | 0.94                       | 5.27                  | 9.50             |
| DLiPC               | 220-22-10            | -58,686                 | 16.44 - 40.00                              | 23.56         | 1.17                       | 5.08                  | 9.50             |
|                      | 220-33-10            | -58,421                 | 29.60 - 40.00                              | 10.40         | 1.18                       | 4.94                  | 9.00             |
|                      | 220-66-10            | -56,569                 | 29.60 - 40.00                              | 10.40         | 1.04                       | 5.22                  | 9.00             |
|                      | 220-88-10            | -55,778                 | 20.08 - 40.00                              | 19.92         | 0.97                       | 5.37                  | 8.90             |
thickness of the membrane. On the contrary, increasing the amount of cholesterol in the system decreased the area per lipid of the liposome membrane. The phospholipid species that compose the system also influence the liposome size and membrane thickness. Liposome membrane thickness is calculated by measuring the distance between two phosphate head group peaks per phospholipid bilayer (i.e., the peak on the first layer of the phosphate head group and the peak on the second layer of the phosphate head group) and locating in the middle point between them (Guixà-González et al. 2014). As shown in Table 3, the membrane thickness of all simulation systems capable of forming liposomes ranges between 4.36 – 5.43 nm, indicating the maximum membrane thickness that a phospholipid bilayer can achieve when forming liposomes during 40 ns of simulation. Meanwhile, the liposome size obtained in this study ranged from 8.00 – 9.50 nm. As Hudiyanti (Hudiyanti et al. 2014) has explained, the liposome is not a stable self-assembled structure. Its curved bilayer can deform the system into a more stable planar structure as the simulation time increases. Therefore, the liposome size obtained in this study is the smallest size that can be obtained when liposomes are formed during the 40 ns time of the simulation.

The data indicated that, in general, the liposomes size and the thickness of the membrane increased with increasing head groups size (Hudiyanti et al. 2019b) and the number of carbon atoms in the acyl chains. The number of double bonds in each hydrocarbon tail of the phospholipid in the system did not significantly influence the liposomes size, the membrane’s thickness, and the area per lipid of the liposome membrane. Based on the phospholipid head groups from the lowest, the membrane thickness and liposome size were PE < PS < PC, while based on the number of the carbon...
atom and the number of a double bonds in the hydrocarbon tails, the order was 12:0 < 18:2 < 18:1. The thickness of liposome membranes and the size of liposomes are due to the cholesterol condensation effect. The condensation effect arises from the rigid sterol ring of the cholesterol structure that suppresses the density of the phospholipid hydrocarbon tail; the greater the amount of cholesterol in the system, the greater the condensation effect. The phospholipid acyl chains in the liposome bilayer become denser and more regular and increase the thickness of the membrane and the liposome size (Alwarawrah et al. 2010).

## Conclusion

The simulations portray the aggregation process of the system with 6 different phospholipid species, cholesterol, and vitamin C during the 40 ns simulation. At least 6 other aggregate structures formed: curved bilayer, toroidal bilayer, concave micelle, disc-like bilayer, planar bilayer, and liposome. The formation of the aggregated structure is determined by the ratio of cholesterol:phospholipid. The ratio between 15% and 40% will form liposomes regardless of the phospholipid species. Cholesterol is located in the liposome bilayer, while vitamin C is located in the liposomes’ core for all cholesterol composition. This study better understands the cholesterol effect on the liposome formation of various phospholipid species and the encapsulation of vitamin C in liposome structure. The simulation provides the basis for cultivating phospholipid-based drug delivery systems.

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