



Research Article

Theme: Lipid-Based Drug Delivery Strategies for Oral Drug Delivery
Guest Editors: Kishor M. Wasan and Sanyog Jain

The Application of Coarse-Grained Molecular Dynamics to the Evaluation of Liposome Physical Stability

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Abstract. Physical stability is one of critical characteristics of liposome, especially to its clinical application. Vesicle fusion was one of the common physical stability phenomena that occurred during the long storage period. Because vesicle fusion could be easily checked by the change of vesicle size, it was widely applied in the evaluation of liposome physical stability. However, since the method requires the liposome to be placed under certain conditions for long-term observation, a liposome physical stability test usually takes several weeks, which greatly hinders the research efficiency. In this study, to speed up the research efficiency, coarse-grained molecular dynamics was first applied in the study of liposome physical stability. By analyzing the microprocess of vesicle fusion, two parameters including diffusion constant and the total time of the vesicle morphology transition process were employed to study the liposome physical stability. Then, in order to verify the applicability of two parameters, the physical stability of elastic liposomes and conventional liposomes was compared at 3 different temperatures. It was found that the fusion probability and speed of elastic liposomes were higher than those of conventional liposomes. Thus, elastic liposomes showed a worse physical stability compared with that of conventional liposomes, which was consistent with former research. Through this research, a new efficient method based on coarse-grained molecular dynamics was proposed for the study of liposome physical stability.

KEY WORDS: liposome; physical stability; coarse-grained molecular dynamics; vesicle fusion.

INTRODUCTION

The liposome, a closed phospholipid bilayer vesicular system, has received considerable attention as a pharmaceutical carrier of great potential over the past 30 years (1, 2). The ability of liposomes to encapsulate both hydrophilic and hydrophobic drugs, coupled with their biocompatibility and biodegradability, makes liposome attractive vehicles in the field of drug delivery (3).

Physical stability is one of critical characteristics of liposome. It depends on vesicle size and other factors such as surface potential, pH and ionic strength of solution, osmotic pressure difference across the membrane, and fluidity of membrane (4–6). Possible physical stability problems

include loss of entrapped drug, aggregation/fusion of vesicle, and sedimentation (7, 8). Because vesicle fusion could be easily characterized by the change of vesicle size, it has been widely applied in the evaluation of physical stability (7). However, in spite of the convenience, this method requires the liposome to be placed under certain conditions for long-term observation (generally, 1 or 2 months per experiment), which greatly prolongs the development cycle. Therefore, research on liposome physical stability was in need of new, efficient method.

During the last few years, computer simulation has become an important tool for studying the assembly and properties of lipid systems (9–12). Vesicle fusion, as a key step in viral infection (13–16), cellular trafficking (17, 18), and neurotransmitter release (19–21), has also been carried out using computational simulation. What is more, vesicle fusion has been carried out at very different levels of detail, including atomistic molecular dynamics (22), coarse-grained molecular dynamics (CG MD) (23, 24), solvent-free techniques (25), and dissipative particle dynamics (26). As a result, in addition to its important role in the study of fusion mechanism, computer simulation has also been proved to be powerful in investigating the effect of vesicle properties on fusion process, such as lipid constitution (27) and bilayer

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curvature (28). All of these provide guidance for the application of computer simulation in liposome physical stability.

In this study, taking vesicle fusion as a cut-in spot, CG MD was first applied in the research of liposome physical stability. By analyzing the microprocess of vesicle fusion, the evaluation parameters of liposome physical stability were explored at the microlevel. Considering the characteristic of CG MD in efficiency and research scale, the results of this study will not only improve research efficiency dramatically but also deepen our understanding of liposome physical stability.

MATERIAL AND METHODS

Vesicle Fusion Model

There were mainly two types of vesicles involved in this study, including CLs (conventional liposomes) and ELs (elastic liposomes). CLs are simple prototype vesicles with one or more lipid bilayers composed of lipids with or without CHOL (cholesterol). Compared to CLs, ELs are composed of lipids with surfactants, such as SC (sodium cholate). Due to the flexibility conferred on the vesicles by the surfactant molecules, ELs are claimed to show better transdermal permeation effect (29, 30). However, as for the influence of surfactants on vesicle physical stability, it has not been studied yet. Therefore, in this study, while studying the correlation between vesicle fusion and liposome physical stability, we also hope to compare the difference between the two types of vesicles.

Individual vesicle was equilibrated under an isothermal-isobaric (NPT) ensemble with a constant pressure and temperature (1 bar and 310 K, respectively). After 90-ns equilibration, vesicle conformation was extracted out for the building of vesicle fusion model.

In Fig. 1, the vesicle fusion model was consisted of two vesicles in a box of $30 \times 30 \times 50$ nm. In order to simulate the process of spontaneous vesicle fusion, it was necessary to ensure that the two vesicles did not interact before the fusion started. Thus, d_{\min} (the minimum distance between the outer phospholipids of the two vesicles) should be larger than the cutoff distance, which was 1.2 nm in this study.

Four MD simulations of vesicle fusion were performed in this study. The details of the simulations and the nomenclature of the simulation systems are summarized in Table I.

Simulation Details

The lipids and counter ions were modeled using dry martini force field (25). All simulations were performed with versions 4.6.3 of the GROMACS package using a stochastic dynamics (SD) integrator at a time length of 420 ns. The time step and temperature were set to 0.03 ps and 310 K. The simulation interaction cutoff schemes were as follows: van der Waals interactions were smoothly shifted to 0 between 0.9 and 1.2 nm. Coulomb interactions were screened by a relative permittivity constant $\epsilon_r = 15$ and were shifted to 0 between 0.0 and 1.2 nm. This interaction scheme caused the potential and the forces to vanish at the cutoff. The neighbor list was extended to 1.4 nm and updated every 10 steps.

Calculation of Parameters

Before simulating the process of spontaneous vesicle fusion in MD simulations, the initial distance should be set. In this study, the initial distances were mainly controlled using d_{\min} , which was calculated using the integrated toolkit in Gromacs named `g_mindist`.

Two heavy atoms located on different vesicles were considered to be on contact when their distance was shorter than 5 Å. For ions inside vesicle, the cutoff distance was set at 6.5 Å. The cutoff distance was chosen based on the RDF (radial distribution function) of heavy atoms in a single vesicle, by which the first peak was covered. The value of $g(r)$ was calculated using the expression below:

$$g(r) = \frac{N(r)}{4\pi r^2 \rho \delta r} \quad (1)$$

where $N(r)$ is the number of two chosen atoms at a distance r , δr is a spherical shell of thickness at a distance of r of two chosen atoms, and ρ is the number density.

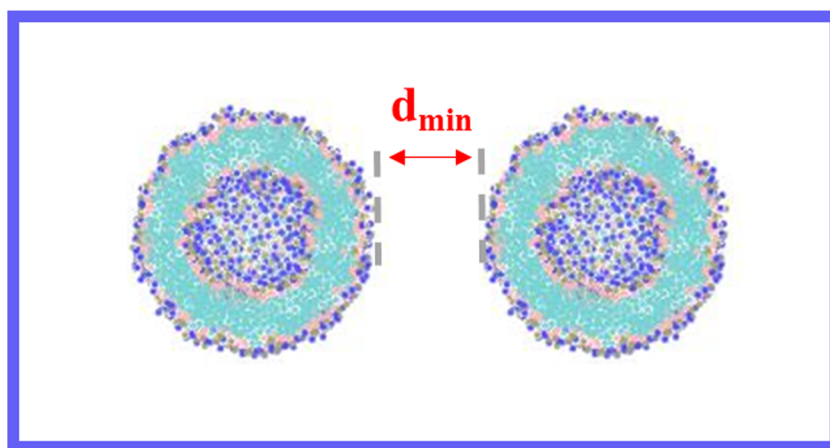


Fig. 1. Graph representing the vesicle fusion model. d_{\min} was the minimum distance between the outer phospholipids of the two vesicles, which were mainly used to control the initial distance between two vesicles

Table I. Names, Compositions, and d_{\min} of the Studied Systems

System name	Atoms	DPPC (inner/outer)	SC (inner/outer)	CHOL (inner/outer)	Na ⁺ (inner/outer)	Cl ⁻ (inner/outer)	d_{\min} (nm)
F-1	13,564	289/548	0/0	108/207	100/400	100/400	1.52
F-2	13,564	289/548	0/0	108/207	100/400	100/400	1.92
F-3	13,564	289/548	0/0	108/207	100/400	100/400	2.65
F-4	13,565	289/548	108/207	0/0	154/504	46/297	1.92

Diffusion constant was employed to characterize the intense of vesicle Brownian motion, and it was calculated in CG MD using the Einstein relation:

$$\text{MSD} = \langle |r_i(t) - r_i(0)|^2 \rangle \quad (2)$$

$$D = \frac{1}{6} \lim_{t \rightarrow \infty} \frac{d}{dt} \sum_{i=1}^{N_a} \langle |r_i(t) - r_i(0)|^2 \rangle \quad (3)$$

where MSD is the average mean square displacement of DPPC beads (NC3 was selected as a representative bead in this study). A total of 2000 configures across 60 ns were used to perform the calculation. It was found that compared with the inner layer of lipids, the outer layer of lipids gave a larger diffusion constant; see Fig. 2. Because the fusion process was triggered by the interaction between outer layers of lipids, the diffusion constant of outer lipids was mainly used to characterize the intense of vesicle Brownian motion.

Δr was the difference between long radius (r_{\max}) and short radius (r_{\min}) and was mainly used to characterize the vesicle morphology transition process from ellipsoid to sphere. It was calculated based on the RDF. r_{\max} was the largest distances of outer lipids to the common mass center of two vesicles. r_{\min} was the smallest distances of outer lipids to the common mass center of two vesicles.

RESULTS AND DISCUSSION

Effect of Initial Distances on Vesicle Fusion

The effect of initial distances on vesicle fusion was first investigated by comparing the vesicle fusion results of F-1, F-2, and F-3; see Fig. 3. According to the difference of the initial distance, the vesicle fusion tests present three different

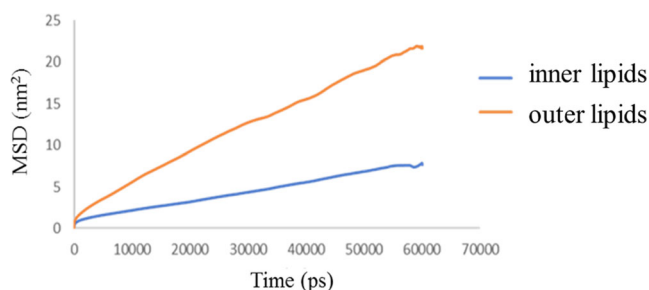


Fig. 2. The diffusion constant of inner and outer lipids of vesicle

phenomena. When the initial fusion distance was large, such as F-3, it was difficult for vesicles to interact with each other. Therefore, the potential energy of the whole system remained stable, and the two vesicles eventually dispersed independently at both ends of the system. When the initial fusion distance was small, such as F-1, the interaction between two vesicles tended to be intense. Instead of vesicle fusion, two vesicles squeezed against each other. Similar phenomena has also been observed in former research (31). Only when the initial distance was within a suitable range, such as F-2, the complete vesicle fusion was observed. Obviously, initial distances showed a great influence to the vesicle fusion results.

Structural Characteristics of Fusion Events

To understand the structural characteristics of vesicle fusion and transformation, taking F-2 as an example, three parameters were calculated as a function of simulation time (see Fig. 4): contact numbers of outer lipids–outer lipids of vesicle pairs (N_{outer}), inner lipids–inner lipids of vesicle pairs (N_{inner}), and contact numbers of inner ions–inner ions of vesicle pairs ($N_{\text{inner ion}}$) (22). *A*, *B*, and *C* were three points when the three parameters were found to change from scratch. At point *A* (100,500 ps), the interaction between outer lipids of vesicle pairs began to take place, which also marked the beginning of fusion. At point *B* (103,500 ps), the interaction between inner lipids of vesicle pairs began to take place. At point *C* (108,300 ps), the interaction between inner ions of vesicle pairs began to take place, which indicated the formation of fusion pore.

In order to have a better understanding of the overall vesicle fusion process, the trajectories of fusion process were projected on two reaction coordinates: the values of N_{inner} and N_{outer} , in terms of the potential mean force. The free energy was calculated using the following equation (22):

$$\Delta G(q_1, q_2) = -k_B T \ln P(q_1, q_2) \quad (4)$$

where q_1 is the value of N_{inner} , q_2 is the value of N_{outer} , and $P(q_1, q_2)$ is a canonical probability distribution function; the lowest free energy was set to 0.

In Fig. 5, from the lower left corner to the upper right corner was the direction of vesicle fusion. It could be seen that except for the two stable states at the beginning and the ending of fusion, the system conformation at point (380, 788) could exist for a long time throughout the fusion process, which could be regarded as an intermediate state of vesicle fusion process.

By observing the fusion trajectory, the intermediate state was thought to be related with the vesicle morphology

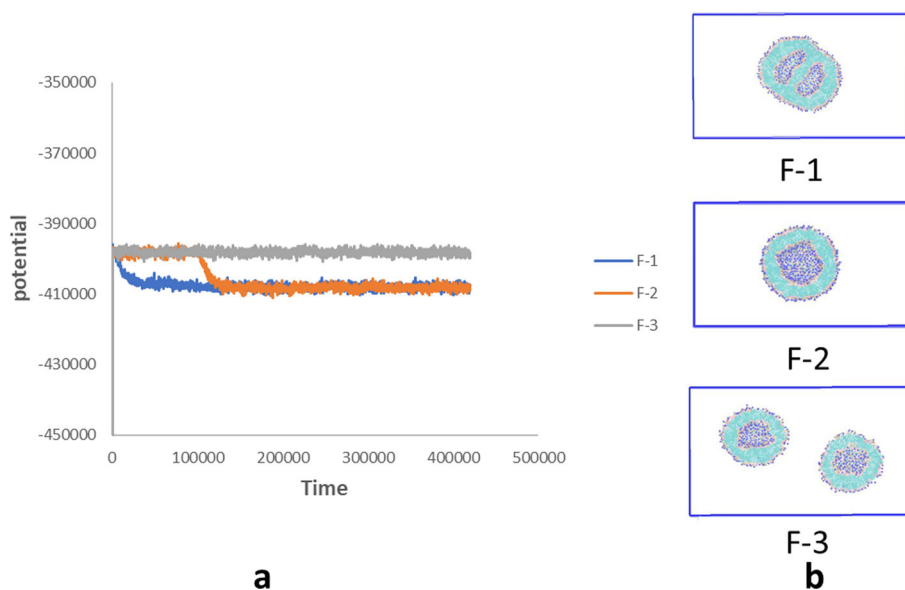


Fig. 3. Potential and vesicle morphology of vesicle fusion for 3 groups with different initial distances. **a** Curve of potential-time. **b** Final vesicle morphology

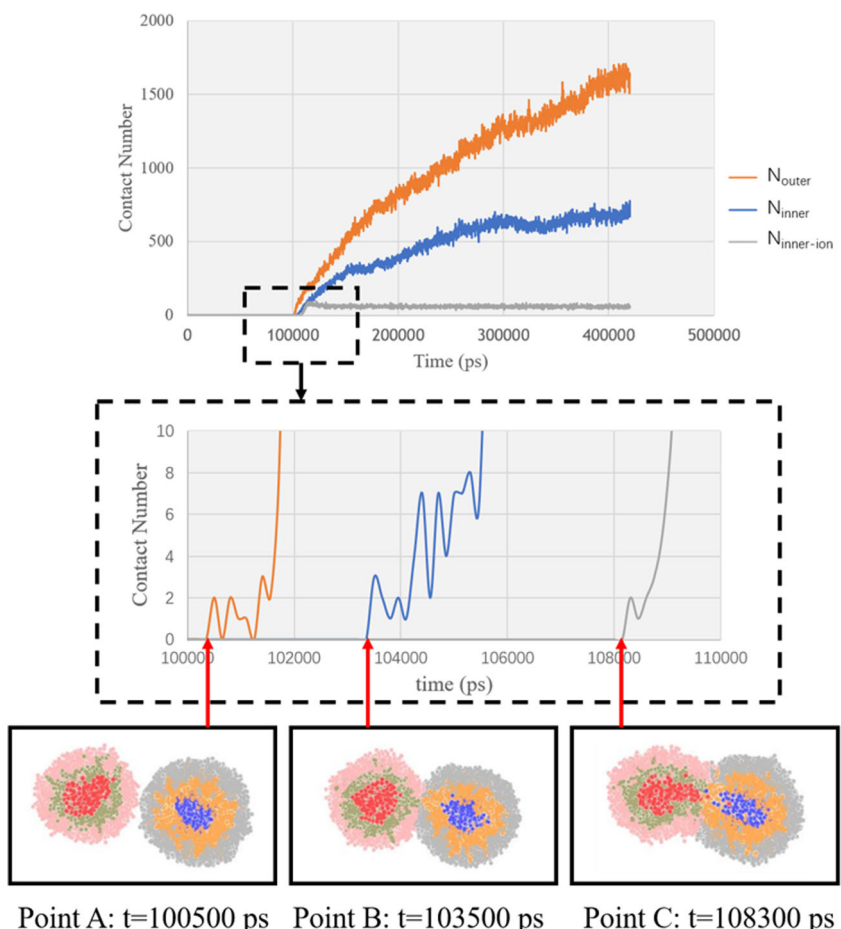


Fig. 4. The curve of N_{outer} , N_{inner} , and $N_{inner-ion}$ with time in the process of vesicular fusion (located at the top of figure). By zooming in the curve at 100,000 ps, A, B, and C were three points when N_{outer} , N_{inner} , and $N_{inner-ion}$ were found to change from scratch (located at the middle of figure). The vesicle morphology of A, B, and C was given at the bottom of figure

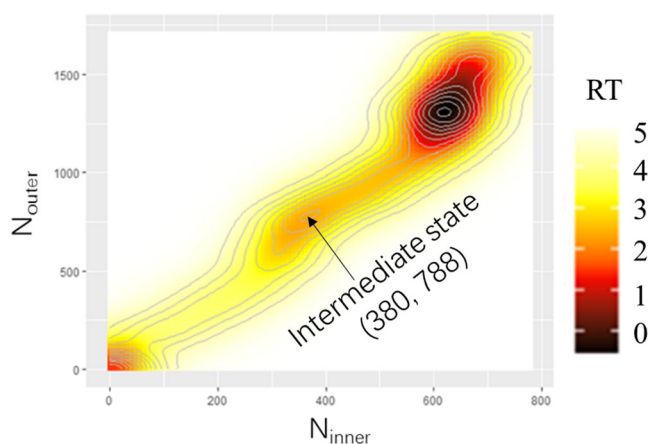


Fig. 5. Free energy landscape of vesicle fusion at 310 K. The free energy landscape is plotted in terms of the values of N_{inner} and N_{outer} . The free energy ranged from 0 to 5 and was painted in different colors. Among these, the lowest free energy was set to 0 and was painted in black

transition process. In this study, a new parameter named Δr was employed to study the vesicle morphology transition process, which was calculated by the difference between long radius (r_{max}) and short radius (r_{min}) of fused vesicle. In Fig. 6a, it was found that as the vesicle fusion started ($t = 100,500$ ps, point A), Δr decreased quickly as the fusion process continues, which indicated that the whole system was undergoing the transformation from ellipsoid to spheroidal.

When $t = 184,500$ ps, Δr hit its platform (~ 2.5 nm) and remained stable. Because the vesicle bilayers were in liquid-crystal phase and presented a high fluidity, a complete symmetry in vesicle structure was hard to be observed. According to the time curve of N_{inner} and N_{outer} , see Fig. 6b, it was found that the system conformation at 184,500 ps was located exactly near point (380, 788). Obviously, the intermediate state and the end of vesicle process were consistent with each other.

Based on the discussion before, the vesicle fusion process was divided into three stages; see Fig. 7. In stage 1, from the beginning of simulation to point A, there was no interaction between two vesicles, and the whole system remained stable. The main behavior of system was irregular Brownian motion. As for stage 1, the probability of vesicle fusion was the key factor that affected the physical stability. Generally, the more intense Brownian motion was, the higher the probability of vesicle fusion would be. Because diffusion coefficient was a common parameter for evaluating Brownian motion in molecular dynamics simulations, it was selected to evaluate the liposome physical stability at this stage.

In stage 2, from point A to the end of vesicle morphology transition, two vesicles began to interact with each other. The main behavior of system was the vesicle morphology transition from ellipsoid to spheroidal. It was worthy noted that stage 2 was also the stage that most molecular dynamics simulation research focused on. Judging from the vesicle morphology and the duration of each state, the whole fusion process observed in this study was similar to former research

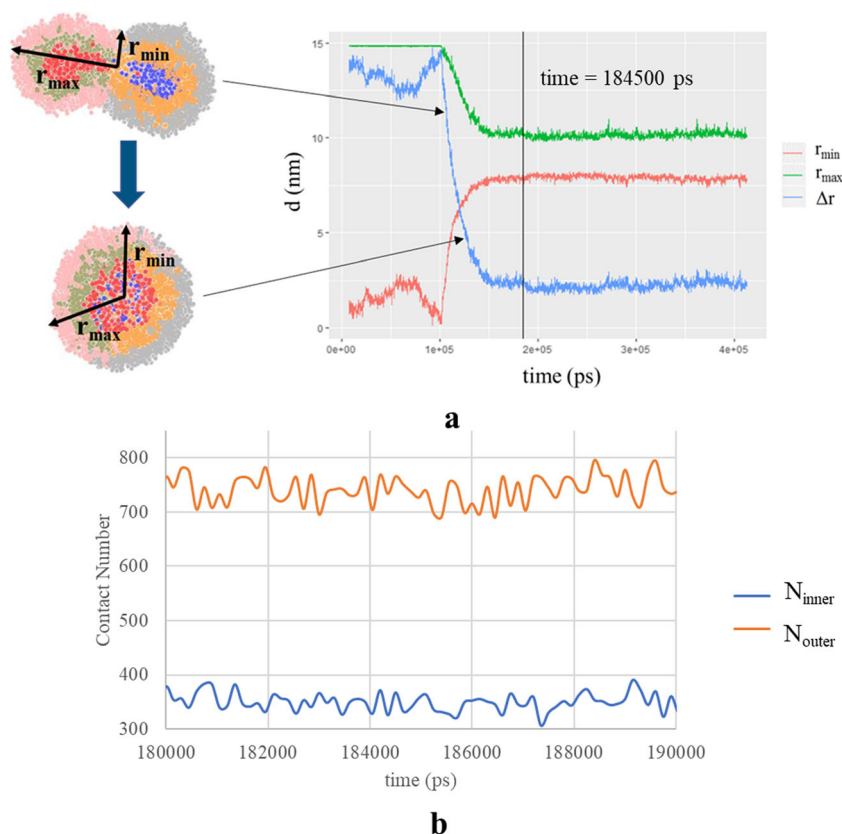


Fig. 6. **a** Three structure properties of vesicle morphology that evolve over simulation time, including r_{min} , r_{max} , and Δr . **b** Time curve of N_{inner} and N_{outer} from 180,000 to 190,000 ps

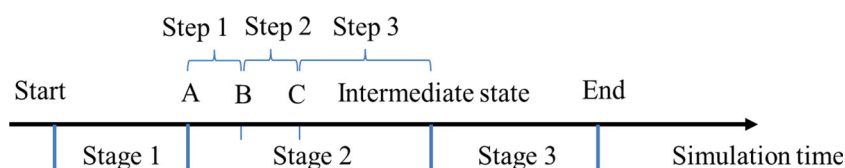


Fig. 7. Decomposition of vesicle fusion process

and was in line with the “stalks-pore formation” mechanism (12, 23). Besides, owing to the application of dry martini force field and the introducing of new parameters (Δr), this work covered a larger time span and provided a more comprehensive analysis on vesicle fusion process. As for this stage, the speed of vesicle fusion process was the key factor that affected the physical stability. Generally, the shorter the vesicle morphology transition lasted, the faster the vesicle morphology transition process took place, the weaker the physical stability would be. Thus, the total time of vesicle morphology transition process was used to characterize the liposome physical stability at this stage. It was further divided into three steps: step 1, from point *A* to point *B*, which was also called “stalk state”; step 2, from point *B* to point *C*, which was also called “hemi-fused”; and step 3, from point *C* to the end of vesicle morphology transition process, which was called “fusing pore.”

In stage 3, from the end of vesicle morphology transition to the end of simulation, the main behavior of system was the redistribution of lipid components. Due to the lack of evaluation parameters, the system behavior at this stage could not be effectively linked to the stability of liposomes. Therefore, the system behavior at this stage will not be discussed.

Comparison of Physical Stability of ELs and CLs at 3 Different Temperatures

To verify the applicability of two selected evaluation parameters, the physical stability of ELs (F-4) and CLs (F-2) was compared at 3 different temperatures. In Table II, the diffusion constant was first compared for these two types of vesicles. It was found that the diffusion constant of both ELs and CLs decreased with the decreasing of temperature, indicating that vesicles were less likely to fuse in a low temperature environment. Comparing the diffusion constant of ELs and CLs, it could be seen that ELs gave a larger diffusion constant, which indicated that EL vesicles tended to be easier to fuse.

Then, total time of vesicle morphology transition process for these two types of vesicles was compared; see Fig. 8. For both types of vesicles, the total time of vesicle morphology transition process increased with the decreasing of tempera-

ture, indicating that the vesicle fusion process had been slowed down. Comparing the diffusion constant of ELs and CLs, it could be concluded that ELs gave a shorter vesicle morphology transition process time, which indicated that the vesicle fusion process between ELs occurred more quickly.

Based on the discussion above, it was found that the result from two parameters, including diffusion constant and total time of vesicle morphology transition process, was consistent with each other. As for the influence of temperature on physical stability, both of the results confirmed that the physical stability of vesicle increased with the decreasing of temperature. As for the difference between ELs and CLs, both of the results confirmed that the introduction of sodium cholate would decrease the physical stability liposome.

Considering this article was an attempt to apply molecular dynamics to vesicle physical stability evaluation, we have tried to compare the results of this study with former physical stability researches (32). As a result, this article has achieved good agreement with previous studies in elucidating the effect of vesicle composition on physical stability. Both confirmed that the introduction of surfactants would reduce the physical stability of liposomes. It proved that coarse-grained molecular dynamics can be used as a qualitative analysis tool for the study of liposome physical stability. As regards the efficiency and economics of molecular dynamics, this study could be regarded as an exploratory work aimed to accelerate the research efficiency of liposome physical stability.

CONCLUSION

In this study, taking vesicle fusion as an entry point, CG MD was first applied in the study of liposome physical stability. By altering the initial distance between vesicle pairs, the entire vesicle fusion process was observed. Then, the entire vesicle fusion process was divided into three stages based on free energy landscape and the trajectory of fusion process. According to the main system behavior at different stages, two parameters, including diffusion constant and total time of vesicle morphology transition process, were used to characterize the physical stability of liposome. By comparing the physical stability of ELs and CLs at 3 different temperatures, the feasibility of CG MD in the evaluation of liposome physical stability was preliminarily proved.

As regards the research scale and efficiency of CG MD, the evaluation method constructed in this study could not only shorten the research cycle of liposome physical stability but also deepen our understanding from the microlevel.

Besides, Δr was first employed in this study to characterize the vesicle morphology transition process. Based on this, studies on vesicle fusion process could be extend from the formation of fusion pore to the end of vesicle morphology transition process, which could be regarded as a supplement and perfection of stalk-pore fusion mechanism.

Table II. Diffusion Constant for ELs and CLs at 3 Different Temperatures

Group	Temperature		
	310 K	298 K	277 K
ELs	0.049 ± 0.006	0.039 ± 0.001	0.028 ± 0.006
CLs	0.037 ± 0.005	0.029 ± 0.006	0.018 ± 0.008

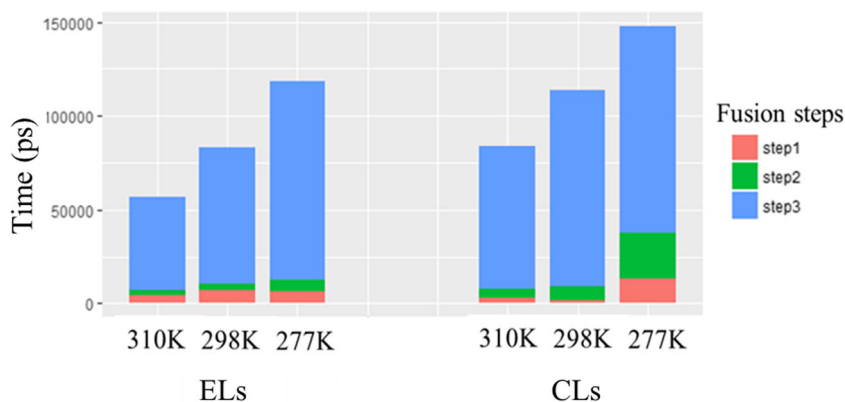


Fig. 8. Total time of vesicle morphology transition process for ELs and CLs at 3 different temperatures

However, owing to the discrepancy between molecular dynamics and classical experiment, we encountered some difficulties in translating the calculated values to measurable quantities: as for molecular dynamics, because the process of vesicle fusion was complicated, the parameters commonly used in the evaluation of the fusion process are microlevel parameters, such as fusion time. Limited by research methods, these parameters were difficult to measure in classical experiments, which made it difficult for us to combine molecular dynamics with experiment.

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