

KYOTO UNIVERSITY

DOCTORAL THESIS

**Studies on dynamics of
functionalized lipid bilayers**

Shunsuke F. Shimobayashi

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Abstract

"What is life?" is one of the most fascinating questions people have. Considering life from a physical point of view, life is a microscale non-equilibrium ordered structure consisting of soft materials, including DNA, RNA, lipids, and proteins. Rich investigations on the physical properties of the soft materials have shed some lights on the physical principals of life. Especially, membrane structure is ubiquitous in life, through which information and substances come in and out. Simplified lipid bilayer vesicles, called liposomes, have been widely used as a model system of cell membrane. Considerable effort has been directed toward elucidating the physics of single liposomes with one- and multi-components and has illuminated some aspects of the physics. However, the underling physics behind functionalized lipid bilayers has been still poorly understood although cell membrane possesses numerous functions, e.g. cell-adhesion through glycolipids and/or proteins. Thus, the effort in this thesis focuses on revealing the physics of functionalized lipid bilayers toward a deeper physical understanding of life.

In Part II, we functionalize multi-component lipid vesicles by externally added glycolipids (GM1: monosialotetrahexosylganglioside), which are extracted from intact cells. The following phase separation dynamics are investigated experimentally and numerically. In Chapter 3, the transition dynamics from macro- to micro-phase separation are experimentally revealed through confocal microscopy for the first time to our knowledge. The transition occurs via stripe morphology as a metastable state. During the transition, monodisperse micro domains emerge through repeated scission events of the stripe domains. In Chapter 4, we numerically confirm the transitions by the time-dependent Ginzburg-Landau model, which describes phase separation and the bending elastic membrane, suggesting that the novel transitions are apparently governed by the local spontaneous curvature induced by the local asymmetric composition. In Chapter 5, we demonstrate the effect of inserted GM1 into lipid bilayers on the bending modulus, to confirm the validity of the significant assumption in the numerical analysis. It is revealed that the bending moduli do not depend on the inserted GM1 in the tested range, guaranteeing the validity of the important assumption. In Chapter 6, based on the expectation by our findings in Chapters 3-5, we quantitatively investigate the relationship between the degree of lipid asymmetry and micro domain size in both of the experiments and numerical analysis, and unexpectedly discover a power law behavior in the relationship.

In Part III, we functionalize single-component lipid vesicles by programmably-synthesized DNA with sticky ends. We experimentally investigate temperature-dependent adhesion of Giant-Unilamellar-Vesicles on supported lipid bilayers mediated by mobile DNA linkers. The simple geometry allows for an accurate characterisation of the morphology of adhering GUVs. For sufficiently high DNA coverage, the adhesion contact angle exhibits a re-entrant temperature dependence. With a single fitting parameter, the model is capable of quantitatively predicting

the low temperature regime. The theory is developed in the limit of strong adhesion, therefore it fails to predict the re-entrant behaviour of the adhesion area, caused by the weakening of the DNA bonds. Moreover, the melting of DNA bonds is investigated in-situ by FRET measurements. We observe a broad melting transition and find that bonds formed within the GUV-plane adhesion patch are more stable than in-plane bonds formed on free bilayers. Our model can semi-quantitatively reproduce these features, although an underestimation of the melting temperature is observed. Furthermore, for the first time to our knowledge, we quantify the temperature-dependent membrane tension induced by DNA bonds by analyzing the thermal fluctuations of the GUVs imaged across their equatorial plane. Membrane tension measurements demonstrate a weak temperature dependence. In a similar range of temperatures, non-adhering GUVs exhibit significantly lower tension, rapidly decreasing upon heating. The differences in magnitude and trend demonstrate the role played by DNA in mediating membrane adhesion.

Our findings in this thesis not only suggest that the functionalization of lipid bilayers to mimic cell membrane functions greatly changes the physical properties of lipid bilayers, but also give us some insight into biological phenomena, which has not been unveiled in the conventional studies on non-functionalized liposomes.

This thesis is based on the following original papers. The chapters which include the contents of the papers are indicated:

Papers under submission

[1] Shunsuke F. Shimobayashi, M. Ichikawa, and T. Taniguchi,
Direct observations of transition dynamics from macro- to micro-phase separation in asymmetric lipid bilayers induced by externally added glycolipids, (2015): Part II [1].

Published papers

[2] Shunsuke F. Shimobayashi, Bartolo M. Mognetti, Lucia Parolini, Davide Orsi, Pietro Cicuta and Lorenzo Di Michele,
Direct measurement of DNA-mediated adhesion between lipid bilayers, *Phys. Chem. Chem. Phys.*, **17**, 15615-15628 (2015): Part III [2].

Reference papers

[3] Shunsuke F. Shimobayashi, Takafumi Iwaki, Toshiaki Mori and Kenichi Yoshikawa,
Probability of double-strand breaks in giant-sized DNA by γ -ray decreases markedly as the DNA concentration increases, *J. Chem. Phys.*, **138**, 174907 (2013) [3].

[4] Shunsuke F. Shimobayashi and M. Ichikawa,
Emergence of DNA-encapsulating liposomes from a DNA-lipid blend film, *J. Phys. Chem. B*, **118**, 10688-10694 (2014) [4].

[5] Tsutomu Hamada, Rie Fujimoto, Shunsuke F. Shimobayashi, Masatoshi Ichikawa and Masahiro Takagi,
Molecular behavior of DNA in a cell-sized small compartment, *Phys. Rev. E*, **91**, 062717. (2015) [5].

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Part I

General introduction

Chapter 1

introduction

In this part, the perspective of this thesis is shown. The thesis includes mainly two topics: "Direct observations of transition dynamics from macro- to micro-phase separation in asymmetric lipid bilayers", and "Direct measurement of DNA-mediated adhesion between lipid bilayers". The significance of this thesis is described. Then, the outline of the thesis is shown.

1.1 Perspectives

"What is life?" is one of the most fascinating questions human beings continue to have. Considering life from a physical point of view, we would say that life is a microscale ordered structure in non-equilibrium conditions, which is composed of numerous kinds of soft materials, including DNA, proteins, and lipid membranes. Rich varieties of spatio-temporal ordered structures in non-linear and non-equilibrium conditions have intensively been researched. Experimental, theoretical, and numerical studies have given us insights of the underlying mechanisms using simple model systems, such as BZ reaction-diffusion [6–8] and Rayleigh Benard convection systems [9, 10]. The developments of physics in non-linear and non-equilibrium systems have shed some light on the physical principals in life [8, 11–15]. On the other hand, numerous investigations on the physical properties of soft biomaterials in life have also illuminated some aspects of physics in life, highly relating to soft matter physics [16–22]. Especially, membrane structure is ubiquitous in life, as known by a lot of organelles enclosed by membrane structure, e.g. mitochondria, and plays biologically important roles through which information and substances come in and out. Therefore, the physics of cell membranes is of fundamental importance in physically understanding life. However, cell membranes are quite complex, composed of many kinds of amphiphile molecules doped with proteins, as shown in FIG. 1.1. Because of the complexity, it is difficult to clearly elucidate quantitative information obtained from intact cells. How should we tackle the problem? One of the most powerful approaches is called as "*bottom-up approach*". In the approach, life-mimicking materials are reconstituted from a small number of cell components. This reduction of variables helps us understanding the observed phenomena clearly and quantitatively. The simplest model system of cell membranes is lipid vesicle, called "*liposomes*". There is a long history on the physics of liposomes. In the early 1970s, Singer and Nicolson proposed the fluid mosaic

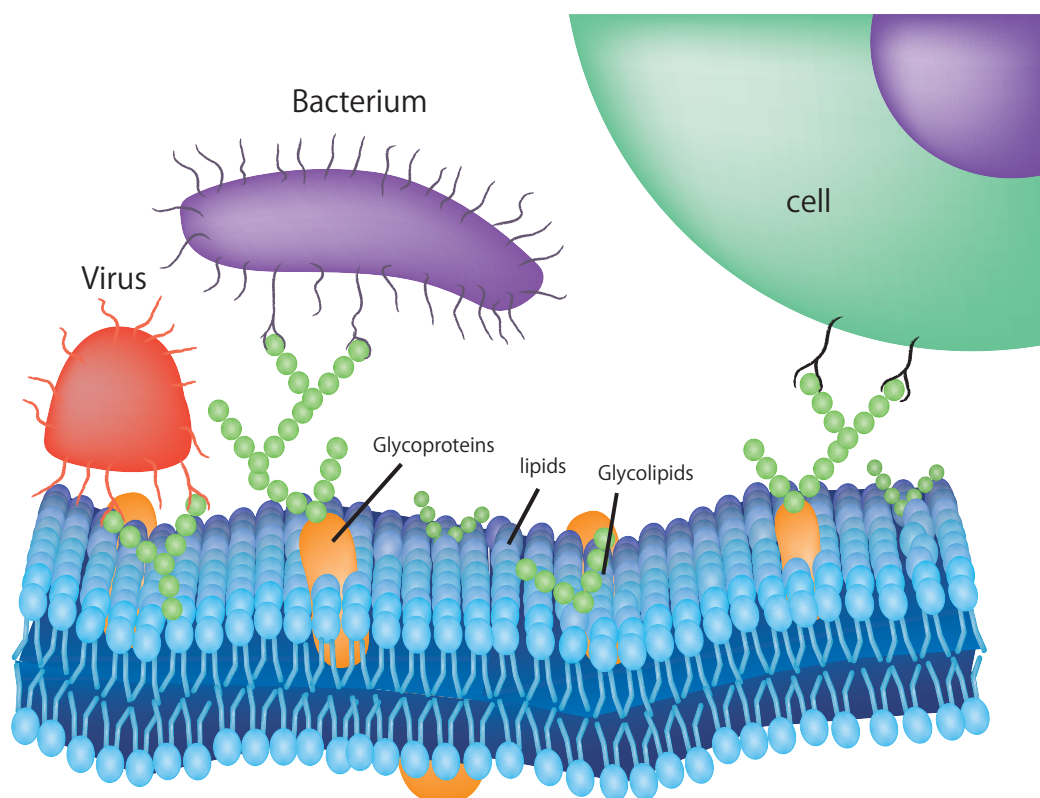


FIGURE 1.1: Schematic illustration of cell membrane structure.

model as an explanation for the data regarding the structure and thermodynamics of cell membranes [23]. Around the same time, Canham [24] and Helfrich [25] proposed slightly different free energy functions of single-component lipid bilayers, respectively. In the early 1990s, the mechanical properties of lipid membrane, such as membrane tension and bending rigidity, have begun to be accessible using by flickering analysis by E. Sackman *et al.* [26] and micropipette aspiration by E. Evans *et al.* [27, 28]. For the two decades, single-component lipid bilayer vesicles have been mainly used as a model system of cell membrane [24–29]. However, in 1997 Simons and Ikonen updated the fluid mosaic model to account for new observations regarding the heterogeneities of lateral diffusion of membrane components, which is named as raft model [30]. The proposal and developments of experimental techniques accelerated the theoretical, numerical, and experimental studies of multi-component lipid vesicles, as represented by the pioneer works of Taniguchi [19] and Baumgart *et al.* [18]. Therefore, there is rich knowledge regarding to the physics of single lipid vesicle with one- and multi-components [18–22, 24–30]. However, the underlying physics behind functionalized lipid vesicles has been still veiled although cell membrane possesses numerous functions, e.g. cell-adhesion, signal transduction, and substance transport through glycolipids¹ [31] and/or proteins [32–34]. The understanding of the physics behind functionalized lipid bilayers is of fundamental importance in physically understanding life [35, 36]

¹lipids with a carbohydrate attached by a glycosidic bond

and could open a new class of functional materials [37]. Therefore, the main topic in this thesis is focused on the physics of functionalized lipid bilayers. Moreover, we focus on the cell-adhesion of the numerous functions. We firstly functionalize multi-component lipid vesicles by externally added glycolipids (GM1) extracted from intact cells. The following phase separation dynamics is investigated experimentally and numerically. Secondly, single-component lipid vesicles are functionalized by programmably-synthesized short DNA with sticky ends. We investigate membrane dynamics and its temperature-response of the functionalized lipid vesicles adhered to glass-supported lipid bilayers due to complementary sticky DNAs.

1.2 Outline of the thesis

In this section, the outline of this thesis is described.

In Chapter 2, two important backgrounds for this thesis are introduced: "Physics of lipid bilayers" and "Physics of phase separation". This thesis has mainly two topics: "Direct observations of transition dynamics from macro- to micro-phase separation in asymmetric lipid bilayers", and "Direct measurement of DNA-mediated adhesion between lipid bilayers". These two topics correspond to Parts II and III, respectively.

Part II includes four chapters. In Chapter 3, we show the first direct observations of morphological transitions from macro- to micro-phase separation in multi-component asymmetric GUVs exposed to externally added glycolipids (GM1). In Chapter 4, the mechanisms of the experimental results in Chapter 3 are investigated by numerical analysis based on the time-dependent Ginzburg-Landau model to describe phase separation and bending elastic membrane. In Chapter 5, to confirm the validity of an important assumption in the numerical analysis, the effect of GM1 on the bending elastic modulus of lipid membrane is investigated. In Chapter 6, an expectation by Chapters 3-5 is confirmed experimentally that the micro domain size should become smaller with the increasing asymmetry of the lipid composition. The contents of this part are under review.

In Part III, we experimentally investigate DNA-mediated adhesion of giant unilamellar vesicles on supported bilayers with the establishment of new experimental protocol. An accurate characterization of the morphology of adhering GUVs and the temperature dependent fraction of bound DNA tethers are shown through confocal microscopy. Finally, the membrane tension induced by DNA bonds is investigated by analyzing the thermal fluctuations of the GUVs. The contents of this part are published in [2].

The conclusion and future problem in this thesis are exemplified in Part IV.

Chapter 2

Background

In this chapter, two topics are introduced, which would assist to understand the investigated results in this thesis: "Physics of membrane" and "Physics of phase separation".

2.1 Physics of membrane

Differential geometry of curved surfaces

Approximately four orders of magnitude separate the thickness of the bilayer (~ 4 nm) from the radius of giant unilamellar vesicles (larger than a few micrometers). This separation enables the description of lipid membranes as a two-dimensional curved surface in three-dimensional space. Therefore, we introduce differential geometry of curved surfaces, which allows us the mathematical description of lipid membranes. First, we consider a curved surface defined by $\vec{r}(u_1, u_2)$, where u_1 and u_2 are arbitrary internal coordinates (FIG. 2.1). For mathematical definitions of the line element ds , area element dA , mean curvature H , and Gauss curvature K , we consider the two tangential vectors along the coordinates at an arbitrary point (u_1, u_2) as follows [38]:

$$\vec{r}_{u_1} = \partial \vec{r} / \partial u_1 = \vec{g}_1, \vec{r}_{u_2} = \partial \vec{r} / \partial u_2 = \vec{g}_2. \quad (2.1)$$

From the two tangential vectors, we obtain the metric tensor g_{ij} as follows:

$$g_{ij} \equiv \vec{r}_{u_i} \cdot \vec{r}_{u_j} = \begin{pmatrix} \vec{r}_{u_1} \cdot \vec{r}_{u_1} & \vec{r}_{u_1} \cdot \vec{r}_{u_2} \\ \vec{r}_{u_2} \cdot \vec{r}_{u_1} & \vec{r}_{u_2} \cdot \vec{r}_{u_2} \end{pmatrix}. \quad (2.2)$$

The distance ds between two points, (u, v) and $(u + du, v + dv)$, is given (to first order) by:

$$\begin{aligned} (ds)^2 &= (\vec{r}_{u_1} du_1 + \vec{r}_{u_2} du_2)^2, \\ &= \vec{r}_{u_1}^2 (du_1)^2 + 2\vec{r}_{u_1} \cdot \vec{r}_{u_2} du_1 du_2 + \vec{r}_{u_2}^2 (du_2)^2. \end{aligned} \quad (2.3)$$

The area element dA is given by:

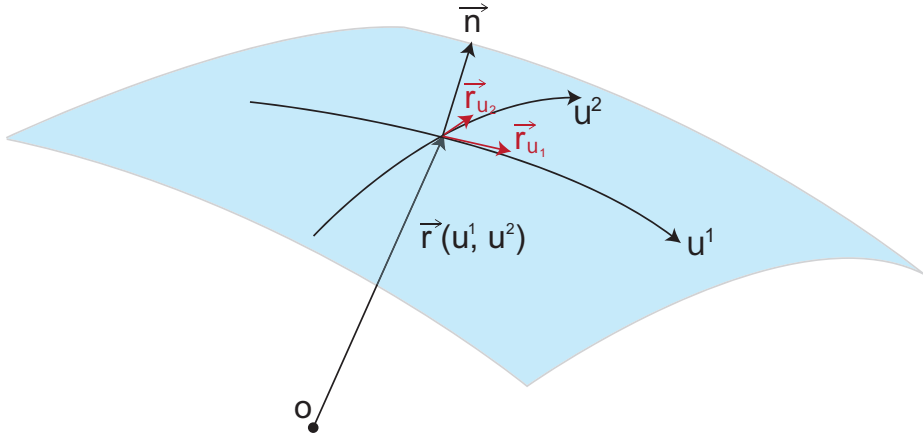


FIGURE 2.1: A membrane portion treated as an infinitely thin sheet. The tangential vector $\vec{r}_{u_\alpha}$ and unit vector $\vec{n}$ normal to the surface are shown.

$$\begin{aligned} dA &= |\vec{r}_{u_1} \times \vec{r}_{u_2}| du_1 du_2, \\ &= \sqrt{g} du_1 du_2, \end{aligned} \quad (2.4)$$

where

$$g = \det(g_{ij}). \quad (2.5)$$

Using the normal vector $\vec{n}(u_1, u_2)$ at the point (u_1, u_2) , we can obtain the curvature tensor h_{ij} :

$$h_{ij} \equiv (\partial_i \partial_j \vec{r}) \cdot \vec{n}, \quad (2.6)$$

where the normal vector $\vec{n}(u_1, u_2)$ is given by:

$$\vec{n} = \frac{\vec{r}_{u_1} \times \vec{r}_{u_2}}{|\vec{r}_{u_1} \times \vec{r}_{u_2}|}. \quad (2.7)$$

The inner product of the inverse of the metric tensor g_{ij} and the curvature tensor h_j^i , $h_j^i \equiv g^{ik} h_{kj}$, gives us the mean curvature H and Gauss curvature K as follows:

$$H \equiv \frac{1}{2} \text{tr}(h_j^i) \quad (2.8)$$

and

$$K \equiv \det(h_j^i), \quad (2.9)$$

where

$$g^{ij} = (g_{ij})^{-1}. \quad (2.10)$$

We now introduce the two following fundamental forms in differential geometry:

- The first fundamental forms: $E = \vec{r}_{u_1} \cdot \vec{r}_{u_1}$, $F = \vec{r}_{u_1} \cdot \vec{r}_{u_2}$, $G = \vec{r}_{u_2} \cdot \vec{r}_{u_2}$,

Monge parameterization

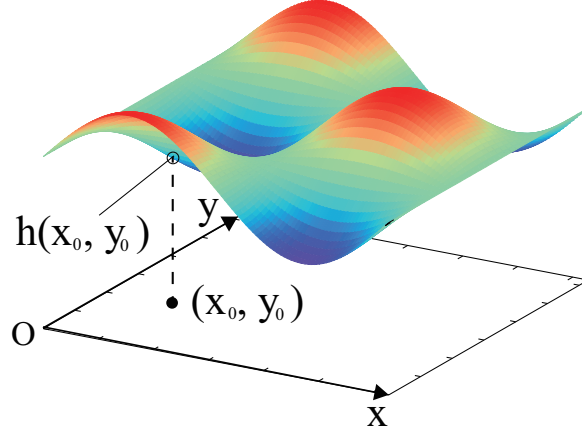


FIGURE 2.2: Schematic illustration of Monge parameterization.

- The second fundamental forms: $L = \vec{r}_{u_1 u_1} \cdot \vec{n}$, $M = \vec{r}_{u_1 u_2} \cdot \vec{n}$, $N = \vec{r}_{u_2 u_2} \cdot \vec{n}$,

where $\vec{r}_{u_1 u_1}$, $\vec{r}_{u_1 u_2}$, and $\vec{r}_{u_2 u_2}$ are defined as $\partial^2 \vec{r} / \partial u_1^2$, $\partial^2 \vec{r} / \partial u_1 \partial u_2$, and $\partial^2 \vec{r} / \partial u_2^2$, respectively. Furthermore, we used the relations, $\vec{r}_{u_1} \cdot \vec{n} = 0$ and $\vec{r}_{u_2} \cdot \vec{n} = 0$. Using the two fundamental forms, ds , dA , g_{ij} and h_j^i are rewritten by:

$$(ds)^2 = E(du_1)^2 + 2Fdu_1 du_2 + G(du_2)^2, \quad (2.11)$$

$$dA = \sqrt{EG - F^2} du_1 du_2, \quad (2.12)$$

$$g_{ij} = \begin{pmatrix} E & F \\ F & G \end{pmatrix}, \quad (2.13)$$

$$\text{and } h_j^i = \begin{pmatrix} E & F \\ F & G \end{pmatrix}^{-1} \begin{pmatrix} L & M \\ M & N \end{pmatrix}, \text{ respectively.} \quad (2.14)$$

Using the first and second fundamental forms, the mean curvature H and Gauss curvature are described;

$$H = \frac{EN + GL - 2FM}{2(EG - F^2)},$$

$$\text{and } K = \frac{LN - M^2}{EG - F^2}, \text{ respectively.} \quad (2.15)$$

The derivation using two principal curvatures is shown in the Appendix A.

We pick one particular useful surface description, *Monge parameterization* (FIG. 2.2). The surface is described by specifying its height $h(x, y)$ above arbitrarily chosen horizontal plane (x, y) . Using Monge parameterization, a point at a surface is

given by $\vec{r} = (x, y, h(x, y))$ and the normal vector $\vec{n}$ at the point is given by:

$$\vec{n} = \frac{\vec{r}_{u_1} \times \vec{r}_{u_2}}{|\vec{r}_{u_1} \times \vec{r}_{u_2}|} = \frac{1}{\sqrt{1 + h_x^2 + h_y^2}} \begin{pmatrix} -h_x \\ -h_y \\ 1 \end{pmatrix}. \quad (2.16)$$

We also can get $E = 1 + h_x^2$, $F = h_x h_y$, $G = 1 + h_y^2$, $L = h_{xx}/\sqrt{1 + h_x^2 + h_y^2}$, $M = h_{xy}/\sqrt{1 + h_x^2 + h_y^2}$ and $N = h_{yy}/\sqrt{1 + h_x^2 + h_y^2}$. By substituting these relations for Eqs. (2.15), the mean curvature H and Gauss curvature K are given by:

$$H = \frac{(1 + h_x^2)h_{yy} + (1 + h_y^2)h_{xx} - 2h_x h_y h_{xy}}{2(1 + h_x^2 + h_y^2)^{\frac{3}{2}}} = \frac{1}{2} \nabla^2 h (1 + \mathcal{O}[(\nabla h)^2]),$$

and $K = \frac{h_{xx}h_{yy} - h_{xy}^2}{(1 + h_x^2 + h_y^2)^2} = (h_{xx}h_{yy} - h_{xy}^2)(1 + \mathcal{O}[(\nabla h)^2])$, respectively. (2.17)

Under the conditions $h_x \ll 1$ and $h_y \ll 1$, H and K are approximately given by:

$$H \simeq \frac{1}{2}(h_{xx} + h_{yy}),$$

and $K \simeq h_{xx}h_{yy} - (h_{xy})^2$, respectively [39]. (2.18)

Bending elastic free energy of lipid bilayers

The pioneer works about modern descriptions of lipid membranes were done by Canham [24] and Helfrich [25] in the early 1970s, who predicted the form of free energy by symmetry arguments with the use of surface invariants, namely the mean curvature H and Gaussian curvature K . Moreover, they assumed that the bending energy should be the quadratic form of the curvatures. Under these assumptions, they predicted the following free energy of bending elastic lipid bilayers. The vesicle is represented by a two-dimensional closed surface parametrized by $u = (u^1, u^2)$. Hamiltonian of a homogeneous lipid vesicle is described as follows, named as the Helfrich's bending elastic energy:

$$H = \frac{\kappa}{2} \int (H - H_{\text{sp}})^2 \sqrt{g} d^2 u + \kappa_G \int K \sqrt{g} d^2 u + \sigma \int \sqrt{g} d^2 u + \int \Pi dV, \quad (2.19)$$

where κ and κ_G are the bending elastic modulus and saddle splay modulus, respectively. H and H_{sp} are the mean curvature and spontaneous curvature, respectively. If the lipid bilayer is up-down asymmetric, the membrane would like to be spontaneously curved. Namely, the spontaneous curvature is non-zero. σ , Π , and V are the surface tension, osmotic pressure difference between the inside and outside of the vesicle, and the vesicle volume, respectively.

Gauss-Bonnet theorem

The second term in the free energy does not depend on the vesicle shape, but depend on the topology. Gauss-Bonnet theorem gives us the following topological invariant $\kappa_G 4\pi(1 - g)$, where g is the genus [29]. In simple terms, the value of the connected surface's genus is equal to the number of the handles the surface has. For instance, the g values of a sphere and torus are zero and one, respectively. Therefore, if we assume a spherical vesicle, the topological invariant is $\kappa_G 4\pi$ because g is zero. Considering one-component lipid vesicle, the second term in the free energy is thus omitted.

Surface tension

Consider the membrane in solution under the condition that the temperature is constant and there is no strong external force. The membrane in solution is always exposed to thermal fluctuations, which slightly increase the membrane area and renormalize the surface tension, conjugated with the membrane area, from its negligible bare value to the effective value [27, 40]. However, the membrane area and surface tension are almost constant unless it is exposed to strong external force because the stretching and compressing of the membrane need much larger energy than bending [37]. In such a case, therefore, the third term is often omitted. Finally, note that we can only measure the renormalized surface tension by thermal fluctuations regardless of the strength of exposed external force [27].

Osmotic pressure

The last term in the free energy is due to the osmotic pressure of the solute molecules dissolved in the solution. Although the membrane is permeable to water, it is impermeable to sugar molecules and ions on the time scale of the experiments (see Table 2.1), which could cause the difference of the osmotic pressure in the inside and outside of the vesicle. The osmotic pressure difference Π is given by:

$$\Pi \equiv \left(\frac{n}{V} - c\right)RT, \quad (2.20)$$

where V is the volume of the vesicle, n is the total number of moles of the solute molecules in the vesicle, c is the total concentration in moles per unit volume in the outer solution, and $R = 8.31 \text{ J (mol K)}^{-1}$ is the gas constant. This osmotic pressure difference gives us the free energy F_{osmo} :

$$F_{\text{osmo}} \equiv \int_{V_0}^V \Pi dV = RT[n \ln(V/V_0) - c(V - V_0)], \quad (2.21)$$

where

$$V_0 \equiv \frac{n}{c} \quad (2.22)$$

is the volume for which osmotic pressure difference becomes zero. Although it is almost impossible to count the solute moles of molecules in a vesicle, the contribution due to osmotic pressure is often ignored except for specific osmotic experiments.

TABLE 2.1: Lipid bilayer Permeability (cm sec^{-1})

	Permeability (cm sec^{-1})
O_2	~ 1
CO_2	$\sim 10^{-2}$
H_2O	$\sim 10^{-2}$
Glycerol	$\sim 10^{-6}$
Glucose, Tryptophan	$\sim 10^{-7}$
Cl^-	$\sim 10^{-10}$
K^+, Na^+	$\sim 10^{-12}$

(http://oregonstate.edu/instruct/bb451/winter14/stryer7/CH12/figure_12_15.jpg)

Parameter measurements of lipid membrane

The mechanical properties in the above-introduced Hamiltonian is accessible using by various methodologies, such as micro-pipette aspiration [27, 28], stretching by optical tweezers [41], analysis of shape flickering [2, 42, 43], and so on. In this thesis, we measure the surface tension and bending modulus of lipid membranes by flickering analysis. Thus, we introduce how the mechanical properties can be measured by flickering analysis, and briefly introduce micro-pipette aspiration method, which is applicable to the higher membrane tension regime.

Flicker spectroscopy of thermal lipid membrane

Shape flickering analysis of thermally fluctuated lipid membranes provides us the main parameters of the membrane mechanics, such as bending modulus and surface tension.

Bending modulus and surface tension

As shown by Eq. (2.19), the bending elastic free energy of one-component vesicles H is described by:

$$H = \frac{\kappa}{2} \int (H - H_{\text{sp}})^2 \sqrt{g} d^2u + \kappa_G \int K \sqrt{g} d^2u + \sigma \int \sqrt{g} d^2u + \int \Pi dV. \quad (2.23)$$

As described above, the second saddle-splay-term is constant (4π) when the Gauss-Bonnet theorem applies for a closed vesicle. In addition, the fourth term is neglected as we assume the no osmotic difference condition. Moreover, we assume

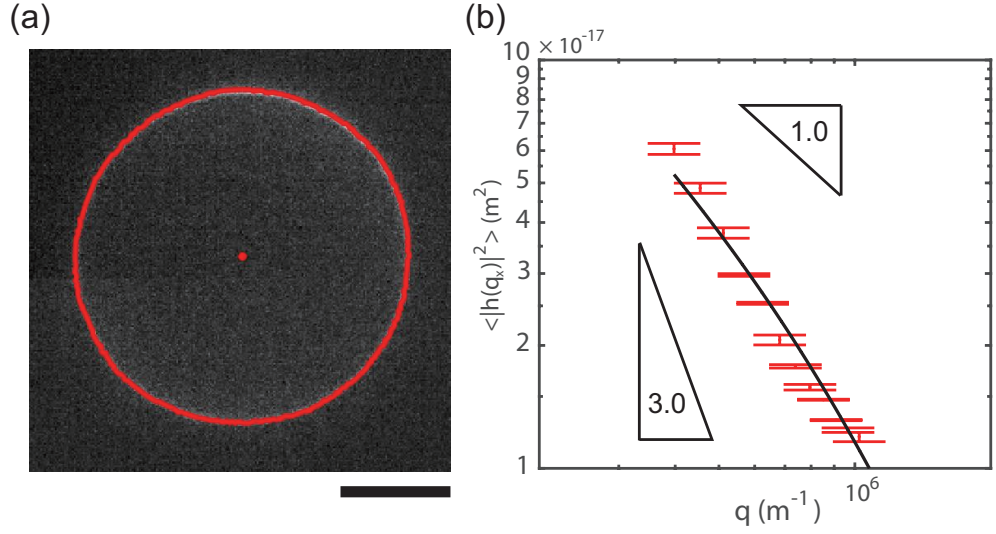


FIGURE 2.3: (a) Contour analysis of a flickering giant vesicle. The vesicle edge (red line) and the center of mass (red dot) are plotted on the equatorial confocal image. Scale bar is $10 \mu\text{m}$. (b) Fluctuation amplitude $\langle |h(q_x, y=0)|^2 \rangle$ is plotted as a function of each q -mode. The data can be fitted by Eq. (2.35) (black-curved line), giving us the values of the surface tension and bending modulus. Limiting behaviors are shown, which are discussed in the text.

the vesicle with the asymmetric lipid composition between the inner and outer layers. In such a case, the hamiltonian is rewritten by:

$$H = \frac{\kappa}{2} \int (H - H_{\text{sp}})^2 \sqrt{g} d^2u + \sigma \int \sqrt{g} d^2u. \quad (2.24)$$

Although a vesicular membrane is curved, it can be regarded as a flat membrane for the the fluctuations in high wave-length regime. Thus, we consider a flat membrane parameterized by the Monge parameterization, with area $A = L \times L$. Then, if there are fluctuations in the membrane hight denoted by $h(x, y)$, the increase in energy due to the work against the surface tension σ is given (to first order) by:

$$\delta E_\sigma = \sigma \int_0^L \int_0^L \frac{1}{2} \left[\left(\frac{\partial h}{\partial x} \right)^2 + \left(\frac{\partial h}{\partial y} \right)^2 \right] dx dy, \quad (2.25)$$

where the surface tension σ is assumed to be constant.

If the membrane has a bending elastic modulus κ , then the energy for bending is:

$$\delta E_\kappa = \kappa \int_0^L \int_0^L \frac{1}{2} \left[\frac{\partial^2 h}{\partial x^2} + \frac{\partial^2 h}{\partial y^2} - H_{\text{sp}} \right]^2 dx dy. \quad (2.26)$$

We now define the 2D Fourier transform as:

$$h(\vec{x}) = \frac{A}{(2\pi)^2} \int_{-\infty}^{\infty} d\vec{q} \tilde{h}_{\vec{q}} e^{+i\vec{q} \cdot \vec{x}}. \quad (2.27)$$

The inverse transform is:

$$\tilde{h}_{\vec{q}} = \frac{1}{A} \int_{-\infty}^{\infty} d\vec{x} h(\vec{x}) e^{-i\vec{q} \cdot \vec{x}}. \quad (2.28)$$

We then get the total energy $\delta E = \delta E_{\sigma} + \delta E_{\kappa}$ due to thermal fluctuation as follows:

$$\begin{aligned} \delta E = \frac{A^2}{2} \int d\vec{x} \left\{ \sigma \left[\left(\int \frac{d\vec{q}}{(2\pi)^2} (iq_x) h_{\vec{q}} e^{i\vec{q} \cdot \vec{x}} \right)^2 + \left(\int \frac{d\vec{q}}{(2\pi)^2} (iq_y) h_{\vec{q}} e^{i\vec{q} \cdot \vec{x}} \right)^2 \right] \right. \\ \left. + \kappa \left[\int \frac{d\vec{q}}{(2\pi)^2} (-q_x^2) h_{\vec{q}} e^{i\vec{q} \cdot \vec{x}} + \int \frac{d\vec{q}}{(2\pi)^2} (-q_y^2) h_{\vec{q}} e^{i\vec{q} \cdot \vec{x}} - H_{\text{sp}}/A \right]^2 \right\} \end{aligned} \quad (2.29)$$

which can be simplified to:

$$\delta E = \frac{A^2}{2(2\pi)^2} \int d\vec{q} \left\{ \sigma(q_x^2 + q_y^2) + \kappa(q_x^2 + q_y^2)^2 \right\} |h_{\vec{q}}|^2 + \frac{H_{\text{sp}}^2 \kappa A}{2}, \quad (2.30)$$

where we used the definition of the delta function:

$$\int d\vec{x} e^{i(\vec{q}_1 - \vec{q}_2) \cdot \vec{x}} = (2\pi)^2 \delta(\vec{q}_1 - \vec{q}_2). \quad (2.31)$$

From equipartition of this energy into modes $\vec{q} = (q_x, q_y)$, we obtain:

$$k_B T = \frac{A}{2} (\sigma q^2 + \kappa q^4) < |h_{\vec{q}}|^2 >, \quad (2.32)$$

where we defined q^2 as $q^2 = q_x^2 + q_y^2$. Therefore:

$$< |h_{\vec{q}}|^2 > = \frac{k_B T}{A} \frac{1}{\sigma q^2 + \kappa q^4}. \quad (2.33)$$

Eq (2.33) was derived from the original work of Helfrich, describing the fluctuations of an 2D membrane. However, in experiments on spherical vesicles, what is observed in the fluorescent microscope is the shape of the equatorial line of each vesicle. Thus, we assume that only modes propagating along the horizontal direction are considered by imaging an equatorial cross sections. For this, we need to transform one of the spatial coordinates back into real space (e.g. take q_y back into y), and fix the value of the spacial coordinate (e.g. take $y = 0$). This gives us the spectrum of the fluctuations of a two-dimensional membrane into that of a one-dimensional line embedded in a two-dimensional plane, allowing us to compare the theoretically derived equation with experimental results. We take q_y back into y and set $y = 0$ as follows:

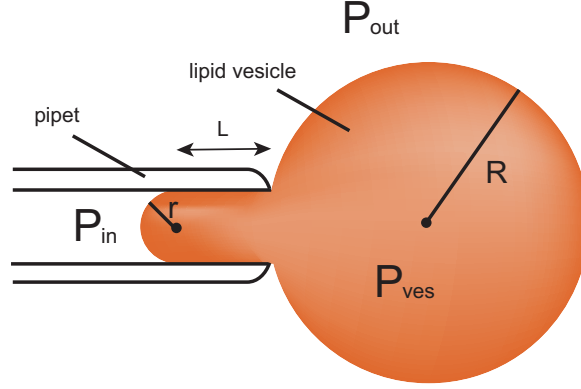


FIGURE 2.4: Schematic of micro-pipette aspiration

$$\begin{aligned}
 \langle h(q_x, y=0)^2 \rangle &= \frac{L}{2\pi} \int dq_y \langle h_q^2 \rangle e^{-iq_y y} \Big|_{y=0} \\
 &= \frac{1}{L} \frac{k_B T}{2\sigma} \left[\frac{1}{q_x} - \frac{1}{\sqrt{\frac{\sigma}{\kappa} + q_x^2}} \right],
 \end{aligned} \tag{2.34}$$

where we used the following relation:

$$\int dx \frac{1}{a(x^2 + y^2) + b(x^2 + y^2)^2} = \arctan\left(\frac{x}{y}\right) \frac{1}{ay} - \arctan\left(\frac{bx}{\sqrt{b(a + by^2)}}\right) \frac{b}{a} \frac{1}{\sqrt{b(a + by^2)}}. \tag{2.35}$$

Eq. (2.34) shows limiting behaviors, which are $\langle h(q_x, y=0)^2 \rangle \sim q_x^{-1}$ for modes dominated by membrane tension ($\sigma \gg \kappa q_x^2$) and $\langle h(q_x, y=0)^2 \rangle \sim q_x^{-3}$ for modes dominated by bending ($\sigma \ll \kappa q_x^2$), as shown in FIG. 2.3. Therefore, what we have to do for measuring the surface tension and bending modulus is (i) to detect the membrane edge, (ii) to calculate the fluctuation amplitudes $\langle h(q_x, y=0)^2 \rangle$ by Fourier transforms, and (iii) to fit the data by the theoretical equation Eq. (2.34), as shown in FIG. 2.3. Also, flickering analysis of the interface line between two phases in multi-component GUVs gives us the line tension, the energy per unit length. The details are shown in Appendix B.

Micro-pipette aspiration

Micro-pipette aspiration of giant unilamellar vesicles is useful for measuring the mechanical properties of area compressibility modulus, surface tension, and bending modulus in single-component GUVs [27, 44], and line tension in multi-component GUVs [45]. In this technique, giant vesicles are aspirated into a glass pipet, whose hole diameter is smaller than the vesicle diameter (FIG. 2.4). Apparent area strain $\alpha = (A - A_0)/A_0$ is measured as a function of the membrane tension σ , where A is the membrane area after increasing the aspiration pressure, and A_0 is the membrane area of the vesicle measured at the lower aspiration pressure. The

relationship is fitted by the following theoretically-derived equation;

$$\alpha = \left(\frac{k_B T}{8\pi\kappa} \right) \ln \left(1 + \frac{c_0 \sigma A}{\kappa} \right) + \frac{\sigma}{K_\alpha}, \quad (2.36)$$

where κ is the bending modulus, K_α is the area compressibility modulus, k_B is the Boltzmann constant, T is the absolute temperature, and c_0 is a constant (~ 0.1) that depends on the type of modes used to describe the membrane fluctuations, respectively [44]. This fitting allows us to measure the bending modulus κ and the area compressibility modulus K_α . The apparent area strain α is obtained by geometrical observables as follows;

$$\alpha = \frac{r^2(1 + L/r)}{2R^2}, \quad (2.37)$$

where R is the vesicle radius, r is the radius of the half sphere in the pipet, L is the length of the aspirated vesicle, respectively (see FIG. 2.4). On the other hand, the membrane tension σ is measured using by the Young-Laplace formula¹;

$$\sigma = \frac{(P_{\text{out}} - P_{\text{in}})r}{2(1 - \frac{r}{R})}, \quad (2.38)$$

where P_{out} and P_{in} are the pressure in the bulk solution and in the pipet, respectively (see FIG. 2.4). Note that the osmotic pressure difference, $P_{\text{out}} - P_{\text{in}}$, can be measured experimentally. Therefore, the relationship between α and σ is experimentally determined.

We briefly introduce the theoretical model. The first term is dominant at small area strains and describes the entropic regime, in which the increase of α is attributable to smoothing out thermal fluctuations of lipid membranes. In this regime, we can obtain the bending modulus κ . On the other hand, the second term is dominant at large area strains and represents the area elastic regime, in which the membrane is directly stretched and lipid density in lipid bilayers is decreased. In this regime, we can get the area compressibility modulus K_α from the slope obtained by fitting the second term to experimental data. Considering the reported results on single-component liposomes, the region of the surface tension, where the two terms compete, is on the order of mN/m [27, 44]. Therefore, note that the micro-pipette aspiration method is applicable to more tensed vesicles than those accessible by usual flickering analysis.

¹ $\Delta P \equiv \sigma(\frac{1}{R_1} + \frac{1}{R_2})$, where $\Delta P = P_{\text{out}} - P_{\text{in}}$ is the pressure difference across the interface, and $1/R_1$ and $1/R_2$ are two principal curvatures at a point in the interface, and σ is the interface tension.

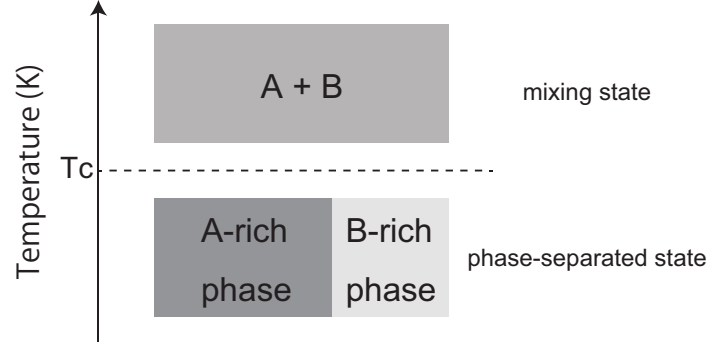


FIGURE 2.5: A schematic of phase separation

2.2 Physics of phase separation

Phase separation

We now introduce a mean-field approximation model to describe phase separation of two immiscible liquid [46]. For simplicity, we first ignore the interface energy. We assume that two types of liquid, A and B , are in a mixing state above a transition temperature, T_c , and separate into two phases below the temperature T_c (FIG. 2.5). We consider how the difference of free energies between the two states, the mixing state and phase-separated state, is dependent on the thermodynamic variables. The mixing free energy, F_{mix} , is defined by:

$$F_{\text{mix}} \equiv F_{A+B} - (F_A + F_B), \quad (2.39)$$

where F_{A+B} and $F_A + F_B$ are the free energies of the mixing state and phase-separated state, respectively. Using mixing energy E_{mix} and entropy S_{mix} , the mixing free energy F_{mix} is obtained by:

$$F_{\text{mix}} = E_{\text{mix}} - TS_{\text{mix}}, \quad (2.40)$$

where T is the absolute temperature.

Mixing energy

We assume that a volume V is divided by virtual lattice cells, whose volumes are denoted by a^d with dimension d . We also assume that either A or B liquid exists at each cell. The total number of lattice cells is $V/a^d (\equiv \Omega)$. We only consider the interaction energy between the nearest molecules. Using the number of the nearest

lattice points of a lattice point z , E_{mix} is given by:

$$\begin{aligned}
 E_{\text{mix}} &= \frac{z\Omega}{2} \left(\phi_A^2 \varepsilon_{AA} + \phi_B^2 \varepsilon_{BB} + 2\phi_A \phi_B \varepsilon_{AB} \right) \\
 &\quad - \frac{z\Omega}{2} \left(\phi_A \varepsilon_{AA} + \phi_B \varepsilon_{BB} \right) \\
 &= \frac{z\Omega}{2} \phi_A \phi_B (2\varepsilon_{AB} - \varepsilon_{AA} - \varepsilon_{BB}) \\
 &= \frac{z\Omega}{2} \phi_A \phi_B \Delta\varepsilon,
 \end{aligned} \tag{2.41}$$

where ϕ_A and ϕ_B are the volume fractions of A and B , respectively. ε_{AA} , ε_{BB} and ε_{AB} are the interaction energies between A and A , B and B , and A and B , respectively. $\Delta\varepsilon$ is defined by $2\varepsilon_{AB} - \varepsilon_{AA} - \varepsilon_{BB}$. A nondimensional parameter, $\chi(T)$, is defined as follows:

$$\chi(T) = \frac{z\Delta\varepsilon}{2k_B T}, \tag{2.42}$$

which is called as χ -parameter. Using $\chi(T)$, E_{mix} is given by:

$$E_{\text{mix}} = \Omega k_B T \chi(T) \phi_A \phi_B. \tag{2.43}$$

Mixing entropy

The configurational entropy S is obtained from Boltzmann's principle as follows:

$$S = k_B \ln W, \tag{2.44}$$

where W is the number of microscopic states. The parameter W is obtained by:

$$W = \frac{\Omega!}{(\Omega\phi_A)!(\Omega\phi_B)!}. \tag{2.45}$$

Using Stirling's approximation $\ln(n!) = n\ln(n) - n + \mathcal{O}[\ln(n)]$, S_{mix} is given by:

$$S_{\text{mix}} = -k_B \Omega [\phi_A \ln \phi_A + \phi_B \ln \phi_B]. \tag{2.46}$$

Mixing free energy

By substituting Eqs. (2.43) and (2.46) into Eq.(2.40), F_{mix} is written by:

$$F_{\text{mix}} = \Omega k_B T [\phi_A \ln \phi_A + \phi_B \ln \phi_B] + \Omega k_B T \chi(T) \phi_A \phi_B. \tag{2.47}$$

Using the relation $\phi \equiv \phi_A = 1 - \phi_B$, the mixing free energy per unit cell $f(\phi)$ is written by:

$$f(\phi) = \frac{F_{\text{mix}}}{\Omega k_B T} = \phi \ln \phi + (1 - \phi) \ln (1 - \phi) + \chi(T) \phi (1 - \phi). \tag{2.48}$$

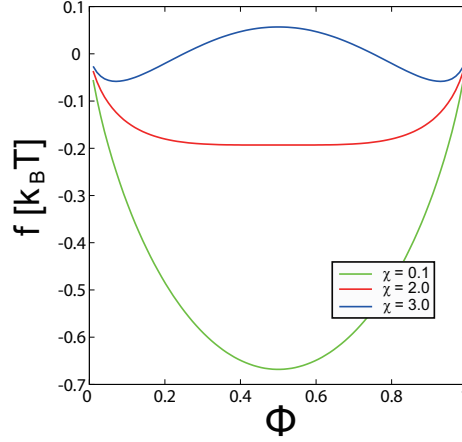


FIGURE 2.6: Dependence of the mixing free energies with three distinct χ -parameters on ϕ .

The mixing free energies $f(\phi)$ with three different χ -parameters ($\chi = 0.1, 2.0$, and 3.0) are plotted as a function of ϕ (see FIG. 2.6). If the value of the χ -parameter is larger than 2.0 , $f(\phi)$ has two local minimums and the system is separated into two phases. Furthermore, we consider how phase separation proceeds. We consider the mixing state of two liquids (A and B), where the volume fraction of A is ϕ_0 . We assume that the solution is separated into two phases, whose volume and its fraction of A are expressed as V_i and ϕ_i ($i \in \{1, 2\}$), respectively. The conservation condition of A molecule number gives us the following relation;

$$\phi_0 = \frac{V_1}{V_0} \phi_1 + (1 - \frac{V_1}{V_0}) \phi_2, \quad (2.49)$$

giving us the relation $V_1/V_0 = (\phi_0 - \phi_2)/(\phi_1 - \phi_2)$. Using this relation, the free energy of the phase separated state per unit cell f_{sep} is given by:

$$f_{\text{sep}} = \frac{(\phi_2 - \phi_0)f(\phi_1) + (\phi_0 - \phi_2)f(\phi_2)}{\phi_2 - \phi_1}. \quad (2.50)$$

Note that the point (ϕ_0, f_{sep}) lies on the line segment connecting the points $(\phi_1, f(\phi_1))$ and $(\phi_2, f(\phi_2))$, and divides the segment in the ratio $\phi_0 - \phi_1 : \phi_2 - \phi_0$ (see FIG. 2.7). Therefore, the mixing state, which locates between two local minimums of free energy, relaxes to the two volume fractions, which are the two contact points of common tangent of free energy $f(\phi)$.

The local stability of ϕ is determined by the sign of $d^2f/d\phi^2$. If $d^2f/d\phi^2 > 0$, the system is locally metastable against small fluctuations. If the large thermal fluctuations occasionally occurred, the nucleation of a droplet occurs and it grows. On the other hand, if $d^2f/d\phi^2 < 0$, the system is locally unstable against small fluctuations, leading to phase separation by spinodal decomposition. The points to satisfy $d^2f/d\phi^2 = 0$ are named as spinodal points, as shown in FIG. 2.7.

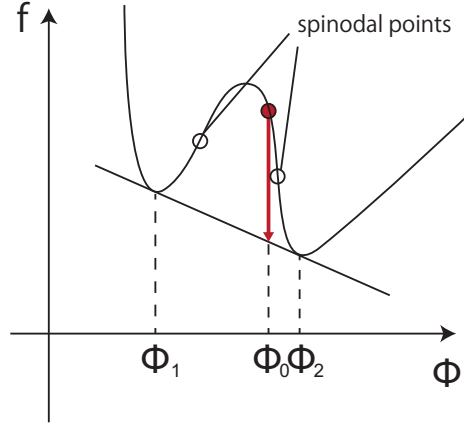


FIGURE 2.7: Free energy vs. volume fraction. The common tangent of free energy and two spinodal points are shown.

Ginzburg-Landau free energy

Fourier expansion of Eq (2.48) with respect to $\psi = \phi - 1/2$ gives us the following result:

$$f(\psi) = (2 - \chi(T))\psi^2 + \frac{16}{3}\psi^4 + \frac{\chi(T)}{4} - \ln 2 + \mathcal{O}[\psi^6]. \quad (2.51)$$

If the value of ϕ is close to $1/2$, the contribution of $\mathcal{O}[\psi^6]$ can be ignored. Moreover, ignoring the constant term, the generic formula of $f(\psi)$ is given by:

$$f(\psi) = a_2\psi^2/2 + a_4\psi^4/4, \quad (2.52)$$

where a_2 is expressed as $|\alpha|(T - T_c)$ and a_4 is the positive coefficient. So far, we did not consider the interface energy due to the composition gradient. Now, we include the interface energy per unit cell using the simplest form $K|\nabla\psi|^2$ with space inversion symmetry, where K is the constant coefficient. Therefore, the total Ginzburg-Landau free energy F_{G-L} in a three-dimensional space can be described as follows:

$$F_{G-L} = \frac{k_B T}{a^3} \int [f(\psi(\vec{r})) + K|\nabla\psi(\vec{r})|^2] d\vec{r}. \quad (2.53)$$

The volume fraction in the equilibrium state is derived from the following equation:

$$\frac{\delta F_{G-L}}{\delta\psi(\vec{r})} = \frac{k_B T}{a^3} [a_2\psi(\vec{r}) + a_4\psi(\vec{r})^3 - 2K\nabla^2\psi(\vec{r})] = 0. \quad (2.54)$$

In the region far from the interface, the interface energy term can be ignored. Thus, the volume fraction in the equilibrium state ϕ_{eq} is obtained as follows:

$$\psi_{eq} = \pm \sqrt{\frac{|a_2|}{a_4}}. \quad (2.55)$$

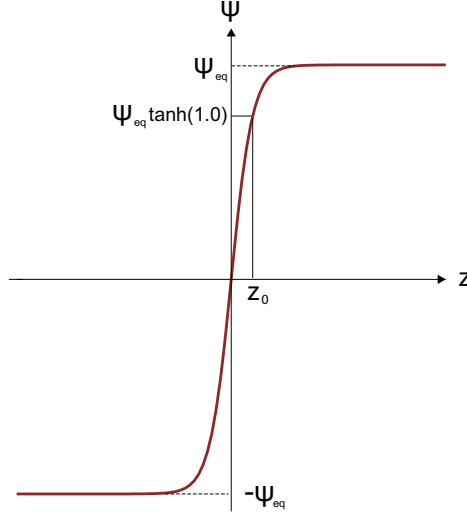


FIGURE 2.8: Interface profile.

Note that the above discussion holds under the conditions that the coefficient a_2 is negative, namely $T > T_c$, and the value of ϕ is close to $1/2$.

Interface profile

We consider an one-dimensional interface profile $\psi(z)$. Setting the boundary conditions that $d\psi(\pm\infty)/dz = 0$, $\psi_{\text{eq}}(+\infty) = \sqrt{|a_2|/a_4}$, and $\psi_{\text{eq}}(-\infty) = -\sqrt{|a_2|/a_4}$ and assuming the position of interface is located at $z = 0$, we can get the following solution (see FIG. 2.8):

$$\psi(z) = \sqrt{\frac{|a_2|}{a_4}} \tanh\left(\frac{z}{z_0}\right), \quad (2.56)$$

where

$$z_0 = \sqrt{\frac{4K}{|a_2|}}. \quad (2.57)$$

The parameter z_0 is called as the interface width (see FIG. 2.8). The interface energy per unit area γ is expressed as:

$$\gamma = \frac{k_B T}{a^3} \int_{-\infty}^{+\infty} dz \left[f(\psi(z)) - f(\psi_{\text{eq}}) + K \left(\frac{\partial \psi(z)}{\partial z} \right)^2 \right] \quad (2.58)$$

Integrating Eq (2.54) along z -axis under the boundary conditions that $d\psi(\pm\infty)/dz = 0$, $\psi_{\text{eq}}(+\infty) = \sqrt{|a_2|/a_4}$, and $\psi_{\text{eq}}(-\infty) = -\sqrt{|a_2|/a_4}$, we get the following relation:

$$f(\psi(z)) - f(\psi_{\text{eq}}) = K \left(\frac{\partial \psi(z)}{\partial z} \right)^2. \quad (2.59)$$

Thus, Eq (2.58) is rewritten by:

$$\begin{aligned}
\gamma &= \frac{2Kk_B T}{a^3} \int_{-\infty}^{+\infty} dz \left(\frac{\partial \psi(z)}{\partial z} \right)^2 \\
&= \frac{2Kk_B T \psi_{\text{eq}}^2}{a^3 z_0^2} \left[\frac{2}{3} z_0 \tanh\left(\frac{z}{z_0}\right) - \frac{1}{3} z_0 \text{sech}\left(\frac{z}{z_0}\right)^2 \tanh\left(\frac{z}{z_0}\right) \right] \Big|_{z=-\infty}^{z=+\infty}, \quad (2.60)
\end{aligned}$$

giving us the interface energy per unit area γ

$$\gamma = \frac{k_B T}{a^3} \frac{4\sqrt{K}|a|^{3/2}}{3a_4} (T_c - T)^{3/2}. \quad (2.61)$$

The mean field theory gives us the critical component with regard to the interface energy γ :

$$\gamma \propto (T_c - T)^{\frac{3}{2}}. \quad (2.62)$$

Finally, defining the other correlation length $\xi_0 \equiv z_0 \sqrt{|a_2|/2}$, Eq (2.53) is converted to the following equation:

$$F_{\text{G-L}} = \frac{k_B T}{a^3} \int \left[f(\phi(\vec{r})) + \frac{\xi_0^2}{2} |\nabla \phi(\vec{r})|^2 \right] d\vec{r}, \quad (2.63)$$

where ψ was replaced by ϕ .

Coarsening dynamics of phase separation: Model A, B, and H

One of the intriguing topics on phase separation is the coarsening dynamics [19, 20, 47–57]. Several descriptions to account for the phase separation dynamics have been proposed, e.g. model A, B, and H, which are classified and named by Hohenberg *et al* [48, 49]. We briefly describe the deferences among the models. In the model A, the order parameter is not conserved but is conserved in the other models. In the model B, the hydrodynamic flow is not included but is included in the model H. The time evolution equations of the order parameter ϕ are defined by:

$$\frac{\partial \phi(\vec{r})}{\partial t} = \begin{cases} -M \frac{\delta F}{\delta \phi(\vec{r})} & \text{(model A),} \\ -\nabla \cdot \vec{J} & \text{(model B),} \\ -\nabla \cdot (\vec{J} + \vec{v} \phi(\vec{r})) & \text{(model H),} \end{cases} \quad (2.64a)$$

$$(2.64b)$$

$$(2.64c)$$

where

$$J_\alpha = -L \frac{\partial \mu}{\partial u^\alpha} + J_\alpha^{(r)}, \quad (2.65)$$

and L , μ , and $J_\alpha^{(r)}(\vec{r})$ are the transport coefficient, the exchange chemical potential, and the random current by thermal fluctuations, respectively. The random current

satisfies the fluctuation dissipation theorem:

$$\langle J_\alpha^{(r)}(\vec{r}, t) J_\beta^{(r)}(\vec{r}', t') \rangle = 2k_B T L \delta_{\alpha\beta} \delta(\vec{r} - \vec{r}') \delta(t - t') / \sqrt{g}. \quad (2.66)$$

Moreover, the fluid velocity $\vec{v}$ in the model H satisfies the Navier-Stokes equation:

$$\rho \left(\frac{\partial \vec{v}}{\partial t} + (\vec{v} \cdot \nabla) \vec{v} \right) = -\nabla p + \eta \nabla^2 \vec{v} + \vec{F}_\phi, \quad (2.67)$$

where ρ , η , and p are the fluid's density, viscosity, and the pressure, respectively. The last term $\vec{F}_\phi$ is the thermodynamic force density acting on the fluid due to the chemical potential gradient. The ϕ - $\vec{v}$ coupling term is given by:

$$\vec{F}_\phi = -\phi \nabla \mu. \quad (2.68)$$

The model A is applicable to describe phase separation of the system with conservation laws, such as Ising model. The model B is used to describe phase separation of the systems, such as binary liquids, binary alloys, and polymer blends, where the hydrodynamics effect is unimportant. The model H is applicable to describe phase separation of the systems, such as binary liquids and polymer blends, where the hydrodynamics effect becomes important. The scaling law of time evolution of the correlation length $\xi(t) \sim t^n$ with the scaling exponent n , which characterizes the coarsening dynamics, is obtained by the real-space pair correlation function of the order parameter $g(r, t) = \langle \phi(\vec{r}, t) \phi(\vec{r}', t) \rangle$ and its Fourier form, namely the structure factor $S(k, t)$. They can be described by,

$$g(r, t) = G(r/\xi(t)) \quad (2.69)$$

and

$$S(k, t) = \xi(t)^d H(k\xi(t)), \quad (2.70)$$

where G , k , d , and H are the space-correlation function, wave number, dimensionality of the system, and time-independent universal scaling functions, respectively [58]. Using numerous systems, including binary alloys, polymer blends and colloids in polymer solution, the exponents n have been revealed theoretically, numerically, and experimentally [19, 20, 47, 54, 55]. The exponents n in the models are as follows;

$$\xi(t) = \begin{cases} \sim t^{\frac{1}{2}} & \text{(model A),} & (2.71a) \\ \sim t^{\frac{1}{3}} & \text{(model B),} & (2.71b) \\ \sim t^{\frac{1}{3}} & \text{(model H: diffusive regime),} & (2.71c) \\ \sim t & \text{(model H: viscous hydrodynamic regime),} & (2.71d) \\ \sim t^{\frac{2}{3}} & \text{(model H: inertial hydrodynamic regime).} & (2.71e) \end{cases}$$

In particular, note that the structure factor $S(k, t)$ can be measured by the time-resolved light scattering experiments, which roughly give us the consistent exponents with theoretical and numerical results [47, 58–60].

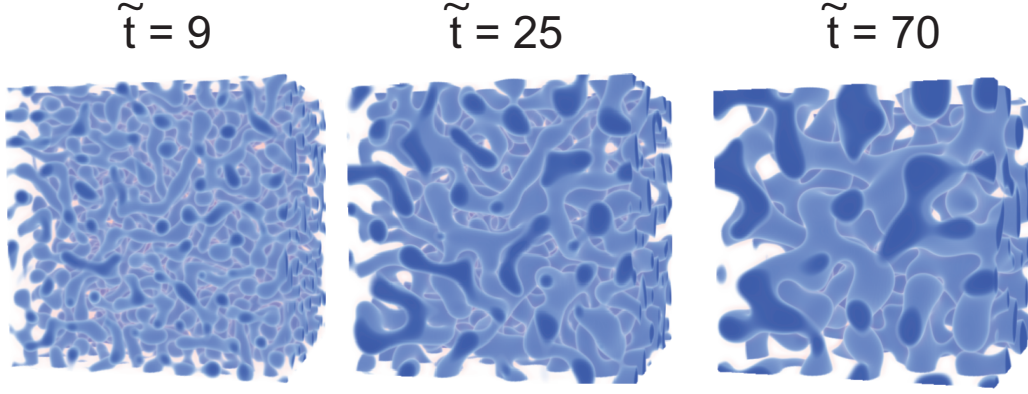


FIGURE 2.9: Three-dimensional phase separation dynamics (model B). One of the two phases is represented. $\phi = 0.3$.

Phase separation on an arbitrary curved surface

To calculate phase separation on an arbitrary curved surface, Differential operators on the surface are introduced in addition to differential geometry in Chapter 2.1. A position specified by u and time t on the surface is given by:

$$\vec{r}(u, t), \quad u \equiv (u^1, u^2). \quad (2.72)$$

The space gradient of a scalar parameter $X(u)$ along the tangential axis is expressed as

$$\nabla X(u) = X_{,\alpha} \vec{g}^{\vec{\alpha}}, \quad (2.73)$$

where $X_{,\alpha}$ denotes the derivative of $X(u)$ with respect to u^α ($\alpha \in 1, 2$), and $\vec{g}^{\vec{\alpha}}$ is the inverse of $\vec{g}_{\vec{\alpha}}$. The divergence of a vector $\vec{X}(u)$ is given by:

$$\nabla \cdot \vec{X}(u) = X_\alpha |^\alpha = X_\alpha |_\beta g^{\alpha\beta} = (X_{\alpha,\beta} - \Gamma_{\alpha\beta}^\gamma X_\gamma) g^{\alpha\beta}, \quad (2.74)$$

where the derivative of $X_\alpha(u)$ with respect to u_β is denoted by $X_\alpha |_\beta = X_{\alpha,\beta} - \Gamma_{\alpha\beta}^\gamma X_\gamma$. The parameter $\Gamma_{\alpha\beta}^\gamma$ denotes the Christoffel symbol and is expressed as $\vec{g}_{\alpha,\beta} \cdot \vec{g}^\gamma$. The Laplace-Beltrami operator Δ_{LB} , used as the Laplacian in a curved space, is written as:

$$\begin{aligned} \Delta_{\text{LB}} X(u) &= X |_\alpha^\alpha = (X_{,\alpha} \vec{g}^{\vec{\alpha}})_{,\beta} \vec{g}^{\vec{\beta}} = X_\alpha |_\beta g^{\alpha\beta} = (X_{\alpha,\beta} - \Gamma_{\alpha\beta}^\gamma X_\gamma) g^{\alpha\beta} \\ &= (X_{,1,1} - X_{,1}\Gamma_{11}^1 - X_{,2}\Gamma_{11}^2) g^{11} + (X_{,2,1} - X_{,1}\Gamma_{21}^1 - X_{,2}\Gamma_{21}^2) g^{21} \\ &\quad + (X_{,1,2} - X_{,1}\Gamma_{12}^1 - X_{,2}\Gamma_{12}^2) g^{12} + (X_{,2,2} - X_{,1}\Gamma_{22}^1 - X_{,2}\Gamma_{22}^2) g^{22}. \end{aligned} \quad (2.75)$$

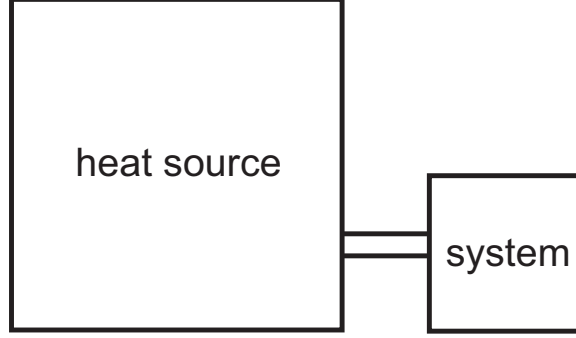


FIGURE 2.10: System in contact with heat source.

Using Eq. (2.63), the free energy of phase separation on an arbitrary curved surface is written as:

$$\begin{aligned} F_{\text{G-L}} &= \frac{k_{\text{B}}T}{a^2} \int [f(\phi(\vec{r})) + \frac{\xi_0^2}{2} |\nabla \phi(\vec{r})|^2] dA, \\ &= \frac{k_{\text{B}}T}{a^2} \int [f(\phi(\vec{r})) + \frac{\xi_0^2}{2} \phi_{,\alpha} \phi_{,\beta} g^{\alpha\beta}] \sqrt{g} d^2u, \end{aligned} \quad (2.76)$$

where we used the relations $\nabla \phi = \phi_{,\alpha} \vec{g}^\alpha$ and $dA = \sqrt{g} d^2u$ (see Eq. (2.4)). Ignoring the hydrodynamic flow, the time evolution of the order parameter $\phi(u)$ on an arbitrary curved surface is given as:

$$\begin{aligned} \frac{\partial \phi}{\partial t} &= L\mu|_\alpha^\alpha - J_\alpha^{(r)}|^\alpha, \\ \mu &= \frac{\delta F_{\text{G-L}}}{\delta \phi} = a_2\phi + a_4\phi^3 - \xi_0^2\phi|_\beta^\beta. \end{aligned} \quad (2.77)$$

Considering the the hydrodynamic flow based on the model H, the time evolutions of the concentration field $\phi(u)$ and velocity field $\vec{v}(u)$ on an arbitrary curved surface are written as:

$$\begin{aligned} \frac{\partial \phi}{\partial t} &= L\mu|_\alpha^\alpha - J_\alpha^{(r)}|^\alpha, \\ \mu &= a_2\phi + a_4\phi^3 - \xi_0^2\phi|_\beta^\beta, \\ \rho \frac{\partial v_\alpha}{\partial t} &= -p_{,\alpha} + \eta v_\alpha|_\beta^\beta - \phi\mu_{,\alpha}. \end{aligned} \quad (2.78)$$

Time-dependent Ginzburg-Landau equation

Finally, we introduce the time-dependent Ginzburg-Landau equation briefly. Consider the system far from equilibrium in contact with heat source, which is a function of an order parameter $m(\vec{r})$. We assume that the system purely relaxes to the equilibrium state to minimize the free energy, and the functional derivative of the free energy with respect to the order parameter $m(\vec{r})$, namely the magnitude of the

gradient $\delta F/\delta m(\vec{r})$, is the force to drive the temporal change of the order parameter. Thus, the time evolution equation of the order parameter is obtained by:

$$\frac{\delta m(\vec{r})}{\delta t} = -\Gamma \frac{\delta F}{\delta m(\vec{r})}, \quad (2.79)$$

where Γ is a kinetic coefficient.

Part II

**Direct observations of transition
dynamics from macro- to micro-phase
separation in asymmetric lipid
bilayers induced by externally added
glycolipids**

Chapter 3

Experimental studies of transition dynamics from macro- to micro-phase separation

3.1 Introduction

Since the proposal of lipid raft concept¹ by Simons and Ikonen in 1997 [30], phase separation in reconstituted multi-component lipid membranes has become a topic of intense research toward revealing the physical principals underlying the nano-sized heterogeneities observed in cell plasma membranes [18, 21, 22, 45, 62–67]. As model systems to investigate the mechanism, the ternary membranes, consisting of DOPC/DPPC/Cholesterol and/or DOPC/Sphingomyelin/Cholesterol, have been mainly used. Following the first reconstitution of liquid-liquid phase separation by C. Dietrich *et al.* [62], reminiscent of the heterogeneities in cell membranes, S. L. Keller *et al.* investigated phase diagrams, miscibility critical points, domain coarsening, and static and dynamic critical exponents [63, 64, 68–70]. T. Baumgart *et al.* measured the mechanical properties, including the bending coefficients and line tension, by fluorescent observation [18], flickering analysis [71], and micro aspiration measurement [45]. P. Cicuta *et al.* unveiled the diffusion constants of liquid-ordered micro domains through fluorescent microscopy and compared them with theoretical predictions by Saffman and Delbrück [65, 72]. M. Yanagisawa *et al.* revealed shape deformation coupled with phase separation using the ternary vesicles with excess area [73]. As described above, although there exists rich knowledge with regard to the physics of the ternary membrane systems, phase separation in adhesive-functionalized lipid vesicles has been poorly understood [74, 75]. In nature, cells interact with other cells and/or extracellular matrix through glycolipids [31] and/or proteins, including selectins, integrins, and cadherins [32–34]. Here, we functionalize the ternary vesicles with the glycolipids because they are easier to treat than proteins. Of the glycolipids, we use GM1 (monosialotetrahexosylganglioside) because it plays a lot of physiologically significant roles, e.g.

¹Recent definition of lipid raft is as follows. Membrane rafts are small (10-200 nm), heterogeneous, highly dynamic, and sterol and sphingolipid-enriched domains that compartmentalize cellular processes, including signal transduction and platforms for virus entry. Small rafts can sometimes be stabilized to form larger platforms through protein-protein and protein-lipid interactions [61].

the recognition sites for lectin family, Cholera toxin and E. coli heat-labile enterotoxin [74, 76]. Moreover, glycolipids exist only in the outer layer of cell membrane and interact with surrounding matrix [77]. Therefore, in this chapter we reveal on phase separation dynamics of multi-component asymmetric lipid vesicles induced by externally added GM1, mimicking the asymmetric distribution of glycolipids in cells.

3.2 Materials and methods

Materials

DOPC (Dioleoylphosphatidylcholine), DPPC (Dipalmitoylphosphatidylcholine), Cholesterol, GM1 (monosialotetrahexosylganglioside), Rho-DOPE (rhodamine-dioleoylphosphatidylethanolamine), and NBD-DPPE (nitrobenzoxadiazole-dipalmitoylphosphatidylethanolamine) were purchased from Avanti Polar Lipids (Alabaster, AL). Texas Red-DHPE (1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt) and Bodipy-GM1 were purchased from Molecular Probes. DOPC, DPPC, Rho-DOPE and Texas Red-DHPE were dissolved in chloroform and stored at -20°C in a freezer. Cholesterol was dissolved in chloroform with methanol (50 % w/w) and stored at -20°C in a freezer. GM1 and Bodipy-GM1 were dissolved in chloroform with methanol (50 % w/w) or Milli-Q water (Millipore) or 400 mM sucrose. GM1 and Bodipy-GM1 were stored at -20°C in a freezer in the first case and at 4°C in a fridge in the other cases. Sucrose and glucose were purchased from Nacalai Tesque, dissolved in Milli-Q water, and filtered through a Millipore syringe filter (Millex-GS, $0.22\ \mu\text{m}$ pore size).

GUV preparation

GUVs were prepared either by electroformation or natural swelling. Natural swelling method is the simplest and easiest method to make GUVs. However, it makes not only GUVs but also some lipid stuffs, and some GUVs are multilamellar. On the other hand, electroformation needs special equipments such as function generator and ITO glass, but almost all GUVs are unilamellar and it hardly makes stuffs.

Natural swelling

Natural swelling method is one of the most standard methods to prepare GUVs, in which the dry lamellar films of phospholipids are hydrated with buffer solutions. DOPC (2.5 mM, $7\ \mu\text{L}$), DPPC (2.5 mM, $7\ \mu\text{L}$), and Cholesterol (2.5 mM, $6\ \mu\text{L}$), including fluorescent dyes (Rhodamine-DOPE (0.5 mol %) or Texas Red-DHPE (0.8 mol %) and/or NBD-DPPE (1 mol %)), were mixed in the bottom of the

glass vial (10 mm × 50 mm, Maruemu Corp. Co. Ltd., Osaka, Japanmm). Moreover, a small amount of NaI was added to generate vesicles with high sphericity [78]. Vesicles with excess area can result in trapped coarsening after temperature quench [21]. Then, the mixed solution was gently dried using nitrogen gas while rotating the vial in-plane centering the long axis at approximate 30 rotation per minute to prepare homogeneous lamellar films. The lipid-coated glass vial is then left to dry under vacuum for at least 1 hour. Milli-Q water or 400 mM sucrose solution was added to the dried lipid film in the bottom of the glass vial. The vial was closed with a poly-cap and sealed with a Parafilm membrane. During the hydration for two hours, the temperature of the solution was kept at 55°C using a chilling/heating plate (Funakoshi) to make the lipid distribution homogeneous. GUVs were recovered by gentle pipetting and used within one week.

Electroformation

DOPC (3.57 mM, 52.5 μ L), DPPC (3.57 mM mM, 52.5 μ L) and Cholesterol (3.57 mM mM, 45 μ L), including fluorescent dyes (Rhodamine-DOPE (0.5 mol %) or Texas Red-DHPE (0.8 mol %) and/or NBD-DPPE (1 mol %)), were mixed in the bottom of the glass vial. Indium Tin Oxide (ITO) coated microscope slides (8-12 Ω /sq, Sigma-Aldrich, MO) were soaked by isopropanol, sonicated at 40°C for 15 minutes, rinsed by Milli-Q water, and sonicated in Milli-Q water at 40°C for 15 minutes again. The solution was spin-coated onto a pre-cleaned ITO coated microscope slide (8-12 Ω /sq, Sigma-Aldrich, MO) at 300 rotation per minute for 2 min. The lipid-coated slide was then left to dry under vacuum for at least 1 hour. Together with a second pre-cleaned ITO coated slide, the lipid-coated slide was arranged to form a capacitor cell, where the two conductive surfaces are facing inwards and were separated by a partially truncated silicone rubber spacer (thickness 0.8 mm, Orion Co., LTD.). The chamber was filled with Milli-Q water or 400 mM sucrose solution degassed in 5-ml syringe (Terumo 5 ml Syringe; Terumo Corp., Tokyo, Japan), using a 27-gauge winged needle 19-mm long and 0.4 mm in diameter (Terumo). The chamber was then sealed with a Parafilm membrane to suppress water evaporation, stabilized with two clips, and connected to a digital function generator (DF-1906, NF Electronics Instruments, Japan). A sinusoidal potential with 1.0 V amplitude (peak-to-peak) is applied with a frequency of 10 Hz for 2 hours and then of 2 Hz for 1 hour. During the hydration, the temperature of the solution was kept at 55°C using a mini-incubator (AsOne, Japan) to make the lipid distribution homogeneous. GUVs were gently recovered using a gel loading tip (1-200 μ L, QSP Liquid Handling Products, USA) at room temperature and used within one week. The experimental setup is shown in FIG. 3.1.

Florescent microscopic observation

Confocal acquisition was performed on an Olympus inverted microscope equipped with a CSU-X1 Yokogawa confocal head and an Andor EMCCD camera. An UplanApo oil ×100/1.35 oil objective lens was used. The system was operated by Andor iQ imaging software (Andor Technology).

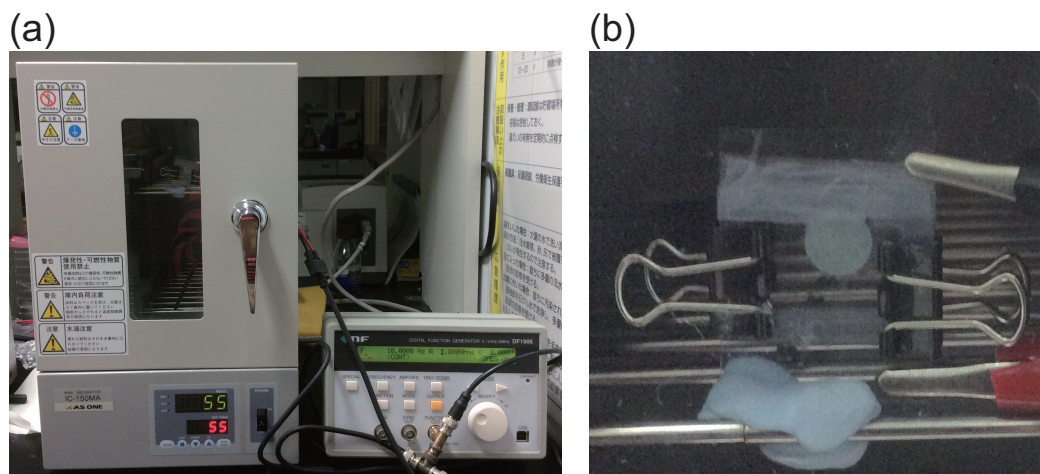


FIGURE 3.1: Experimental setup of electroformation. (a) The mini-incubator (left) and function generator (right) are shown. The chamber in the incubator is connected to the function generator by a coaxial cable. (b) The chamber in the incubator is shown.

3.3 Results

The average radius of the vesicles for natural swelling and electroformation was $4.3 \pm 2.0 \mu\text{m}$ and $6.4 \pm 2.6 \mu\text{m}$, respectively (FIG. 3.2). Subsequently, we decreased the temperature under the miscibility transition temperature, leading to the spontaneous formation of liquid-ordered L_o domains (DPPC rich) in a liquid-disordered L_d matrix (DOPC rich), further coarsening to the fully phase-separated state. GM1 solution was left for at least one hour after ultrasonication. During this time, the GM1 molecules formed a micelle structure with the characteristic time $\tau = 36.0 \pm 13.5 \text{ min}$, as confirmed by dynamic light-scattering measurements (see supplementary information: “Time development of GM1 size after ultrasonication” and Appendix C). The solution of fully phase-separated GUVs was mixed with GM1 solution of varying concentration; $10 \mu\text{M}$ to $250 \mu\text{M}$ (above the critical micelle concentration) [79] (see Appendix D). Intensity measurements using confocal microscopy revealed that GM1 was inserted into the GUVs with a characteristic time of at most 100 minutes (see supplementary information: “Insertion dynamics of GM1 into lipid bilayers by intensity analysis”). The mole fractions of GM1 in the L_d and L_o phases were estimated by comparing the mean intensities of GM1 prepared in the film and introduced from outside (see supplementary information: “Quantitative estimation of the mole fractions of inserted GM1”). The GM1 concentration ratio in the L_d phase to the L_o phase was then estimated as 0.22 ± 0.02 (FIG. 6.2(c)). Finally, the phase-separation transition was identified from the different fluorescent intensities of the incorporated dyes.

Typically, three different types of phase separation morphologies are observed: (a) a fully phase separated morphology (hereafter referring as “macro-phase”), (b) a stripe morphology (“stripe-phase”) and (c) a micro-phase-separation-like morphology (“micro-phase”). L_o domains in the stripe-phase and the micro-phase slightly

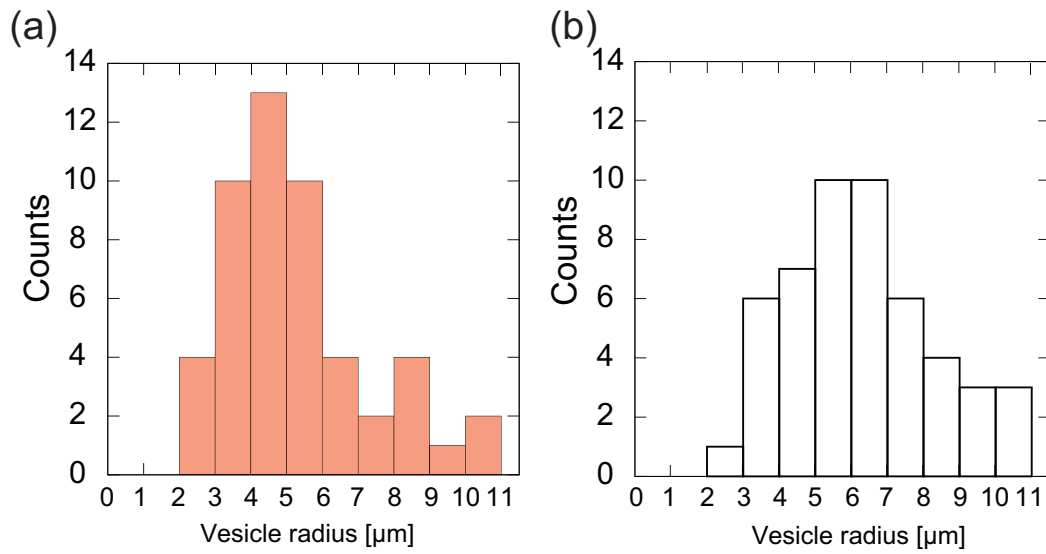


FIGURE 3.2: **Size distribution of GUVs.** (a) The average radius of the vesicles for natural swelling (red closed bars) is $4.3 \pm 2.0 \mu\text{m}$. (b) The average radius for electroformation (black open bars) is $6.4 \pm 2.6 \mu\text{m}$. 50 GUVs were counted.

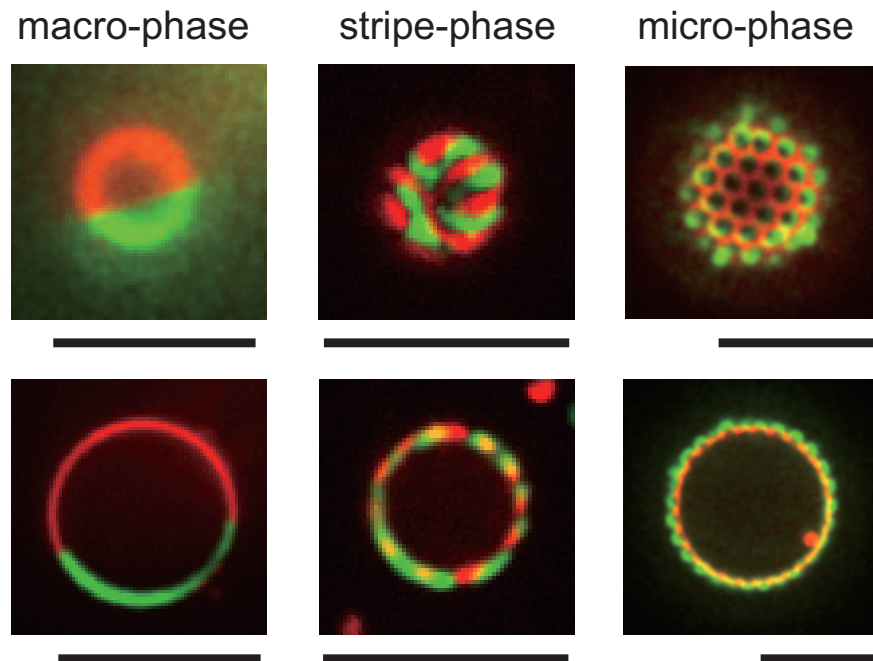


FIGURE 3.3: Phase-separation transition from macro- to micro-phase. Confocal microscope images of top (upper) and cross-sectional (lower) views of GUVs with three different morphologies (macro-phase, stripe-phase and micro-phase). Red and green represent L_d and L_o phases, respectively. The scale bars are 10 μm . (b) Time variation of the fractions of vesicles exhibiting each one of these three phases (solid lines: theoretically fitted curves). Added GM1 concentration is 250 μM .

bud toward the outside of the vesicles. At low L_o domain densities, they diffuse within a fluid L_d matrix. On the other hand, at high densities, they organize into the ordered, largely stationary, crystalline structure of circular domains on the spherical surface (FIG. 3.3). The time evolution of the fraction of vesicles exhibiting each one of these three phases are shown in FIG. 3.4. In these plots, GUVs displaying a coexistence of two different phases were discarded. All of the vesicles were initially the macro-phase state; subsequently, the fraction continuously declined toward zero, while the stripe- and micro-phases emerged.

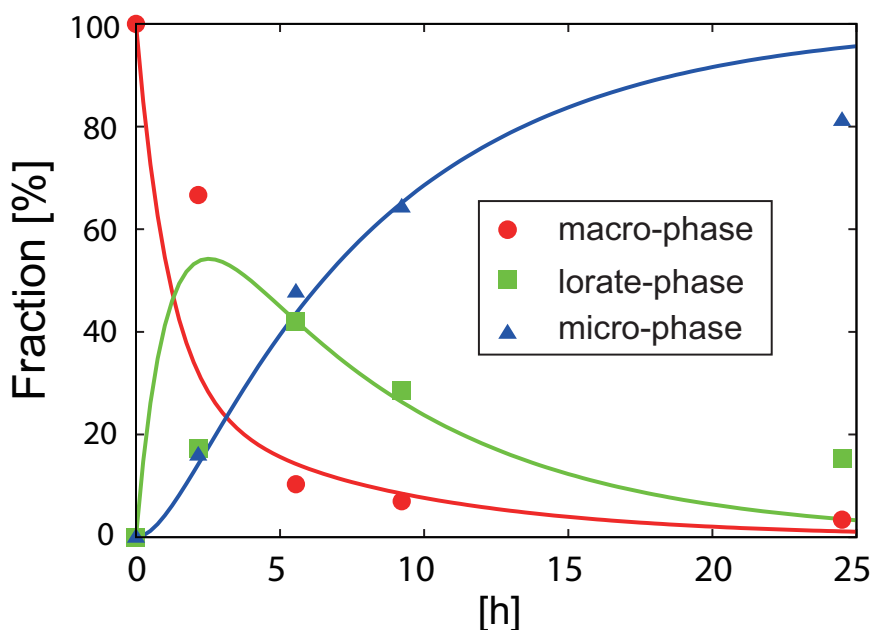


FIGURE 3.4: Phase-separation transition from macro- to micro-phase. Time variation of the fractions of vesicles exhibiting each one of these three phases (solid lines: theoretically fitted curves). Added GM1 concentration is $250 \mu\text{M}$.

The stripe-phase decreased after reaching its maximum at approximately five hours, but the micro-phase continued to increase. These results suggest that the stripe-phase is a metastable state that mediates the transition from the macro- to micro-phase. Therefore, the successive state transitions can be fitted by a pair of coupled differential equations as shown in FIG. 3.4. For this purpose, we employed the Levenberg-Marquardt method (see supplementary information: “Two coupled differential equations describing the successive transition”). We captured the transitions from the macro- to micro-phase via the stripe-phase through confocal microscopy (FIG. 3.5 and 3.7). During the transition process, long wavelength fluctuations developed at the interface between the L_d and L_o phases, inducing the nucleation and growth of stripe domains (indicated by the white arrow in FIG. 3.5). After approximately 10 minutes of successive nucleation-growth events at the fluctuated interface, the macro-phase was completely transformed to the stripe-phase. Moreover, the width of the stripe domain, obtained by dividing the domain area by the thinned line, decreased over time, indicating single-exponential kinetics (FIG. 3.6). Further transition from the stripe- to micro-phases was initiated by a narrow

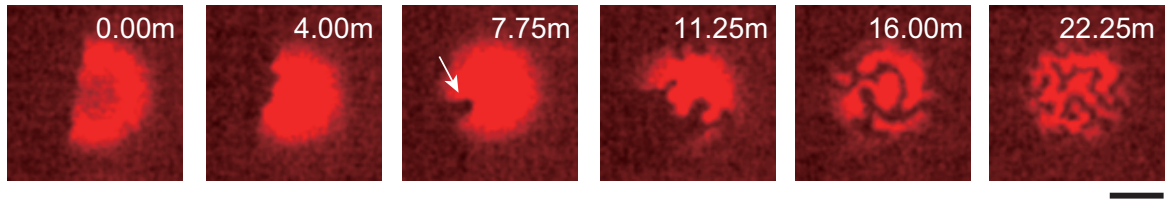


FIGURE 3.5: Transition dynamics from macro- to stripe-phase. Sequential images of top view of a vesicle in the transition from macro- to stripe-phase. Red regions indicate L_d domains. The white arrow shows nucleation of stripe domain. The scale bar is $5\ \mu\text{m}$. Added GM1 concentration is $250\ \mu\text{M}$.

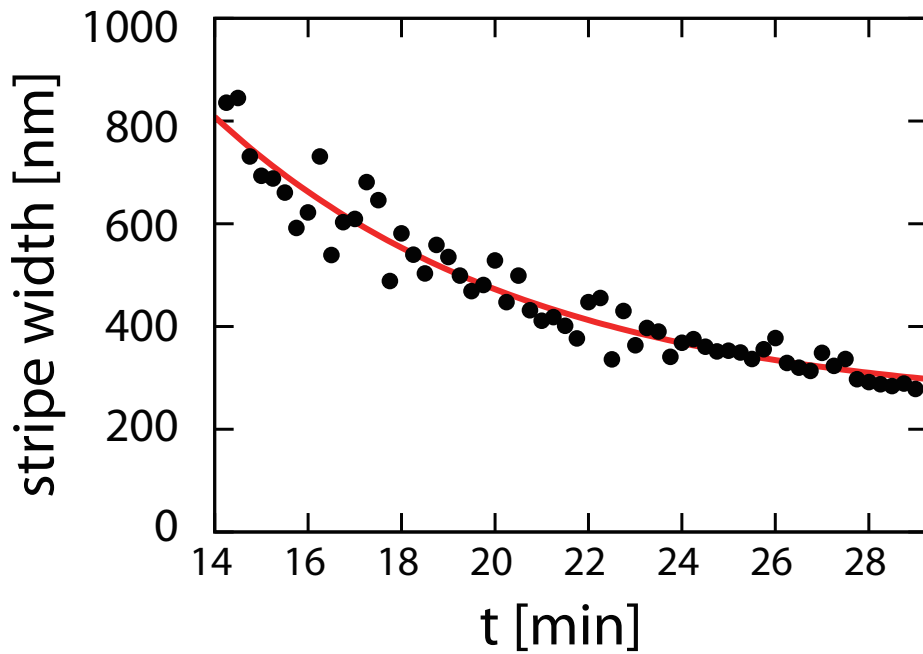


FIGURE 3.6: The time evolution of the stripe width. The time evolution of the stripe width of the vesicle shown in FIG. 3.5, representing single-exponential behavior (red line).

constriction at the tip of a stripe domain, which immediately caused domain scission (the time scale was below 1 s; see white arrows in FIG. 3.7, schematic in FIG. 3.8). After repeated scission events, the stripe domains were completely converted to monodispersed micro-domains.

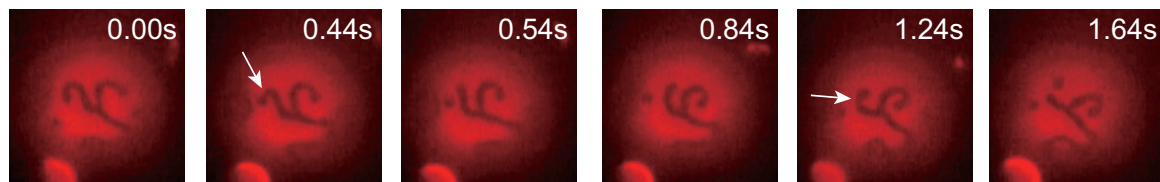


FIGURE 3.7: Transition dynamics from stripe- to micro-phase. Sequential images of top view of a vesicle in the transition from stripe- to micro-phase. The white arrows indicate narrow constrictions emerging immediately before each scission event. The scale bars is 5 μm .

Added GM1 concentration is 250 μM .

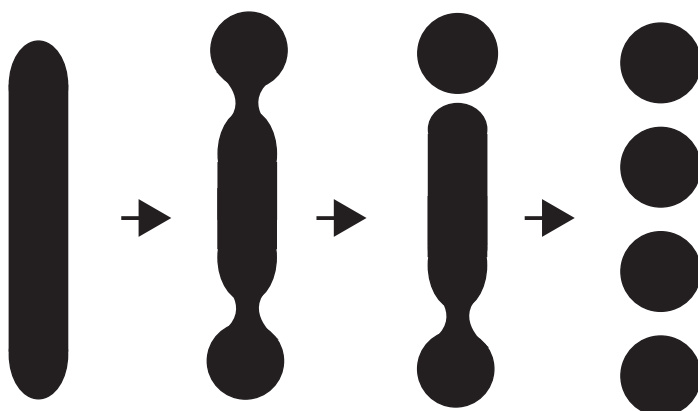


FIGURE 3.8: Schematic illustration of domain scission.

3.4 Discussion

In the normal coarsening process of phase separation in multi-component lipid vesicles, the length of the quasi one-dimensional interface between two different phases decreases to reduce the interface energy. However, in the observed phase separation transitions from macro- to micro-phase via metastable stripe-phase, the interface length continues to increase till the quenched macro-phase relaxes to the micro-phase. This strongly suggests that other parameters are tuned by the GM1 insertion and overcome the contribution by the interface energy. Considering the free energy functional form to describe phase separation coupled with shape deformation of lipid vesicles (see Eqs. (4.1) and (4.2)), the spontaneous curvatures and bending coefficients could be selected as the candidates for the other factors to drive the transitions.

We now discuss the novelty of the observed phenomena to similar reported studies [22, 66]. Quite recently, Scheve *et al.* investigated phase separation in multi-component vesicles with externally adhered proteins, suggesting the molecular doping of proteins transforms phase separation from macro- to micro-phase [66]. Moreover, Yanagisawa *et al.* studied phase separation in multi-component vesicles, where head-bulky lipids (PEG-Cholesterol) are doped with, implying the doped lipids seem to enhance the stability of micro-phase [22]. These similar findings suggest that the molecular doping of proteins or head-bulky lipids modulates stable morphologies of phase separation and the guest molecules enhance the stability of micro-phase separation. However, the transition dynamics from macro- to micro-phase separation by added guest molecules has been veiled. For the first time to our knowledge, we reveal the transition dynamics from macro- to micro-phase separation with micrometer-sized asymmetric lipid vesicles due to externally added glycolipids (GM1). The net novelty of our findings would be the discoveries of the stripe-phase as the metastable phase that mediates the transition from the macro- to micro-phase, and of the novel interface dynamics upon each transition.

3.5 Summary

To summarize, we have revealed the transition dynamics from macro- to micro-phase separation in multi-component asymmetric giant multilamellar vesicles, exposed to externally added glycolipids (GM1:monosialotetrahexosylganglioside). Macro phase with asymmetric lipid composition transits to the micro phase via a metastable stripe-phase. Moreover, during the transition from macro- to stripe-phase, the long wavelength fluctuations developed at the interface between L_o and L_d phases. Subsequently, the nucleation and growth of stripe domains emerged. During the transition from stripe- to micro-phase, monodispersed submicron-sized domains emerge through repeated scission events of the stripe domains.

3.6 Supplementary information

Time development of GM1 size after ultrasonication.

We measured the time evolution of GM1 size after ultrasonication. The hydrodynamic radius of GM1, $R(t)$, is shown as a function of time (FIG. 3.9). Assuming that the GM1 insertion process is the first-order reaction, the data are fitted by the following equation;

$$R(t) = a \left[1 - \exp\left(-\frac{t}{\tau}\right) + b \right], \quad (3.1)$$

giving us the best-fit parameters $a = 4.2 \pm 0.5$ nm, $b = 0.3 \pm 0.2$, and $\tau = 36.0 \pm 13.5$ min. Combining this DLS measurements with the reported X-ray scattering measurements [80], we can conclude that GM1 structure changes from single molecules to micelle structure, with the characteristic time $\tau = 36.0 \pm 13.5$ min (the red-solid line in FIG. 3.9) after ultrasonication, as shown in FIG. 3.10.

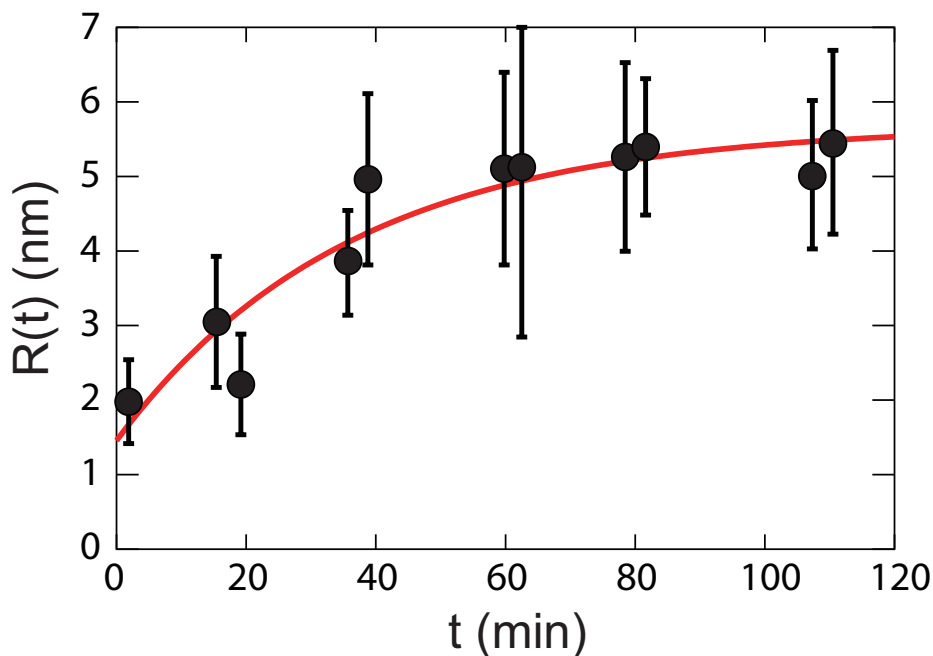


FIGURE 3.9: **Time development of GM1 size after ultrasonication by dynamic light scattering.** The hydrodynamic radii of GM1 exhibits single exponential kinetics with the characteristic time $\tau = 36.0 \pm 13.5$ min (the red-solid line). The measurements were done at room temperature.

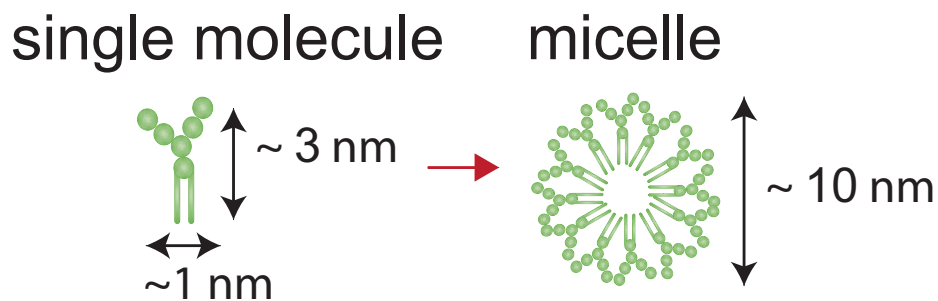


FIGURE 3.10: **Schematic of the time-evolution of GM1 lipids after ultrasonication.**

Insertion dynamics of GM1 into lipid bilayers by intensity analysis.

We investigated the insertion dynamics of GM1 into lipid bilayers by intensity measurements. The fully phase-separated GUVs were mixed with GM1 solution of varying concentration: 10 μM (including 10 mol % Bodipy-GM1), 100 μM (including 1 mol % Bodipy-GM1) and 250 μM (including 1 mol % Bodipy-GM1). The following insertion dynamics of GM1 into L_o and L_d phases was estimated by the time development of the average intensity of Bodipy-GM1 along the equatorial orbits of the GUVs. We have defined the two points, where the intensity of Texas-red (0.8 mol %) indicates an average value along an equatorial orbit, as the boundaries between L_o and L_d phases (FIG. 3.11). Based on the boundary definition,

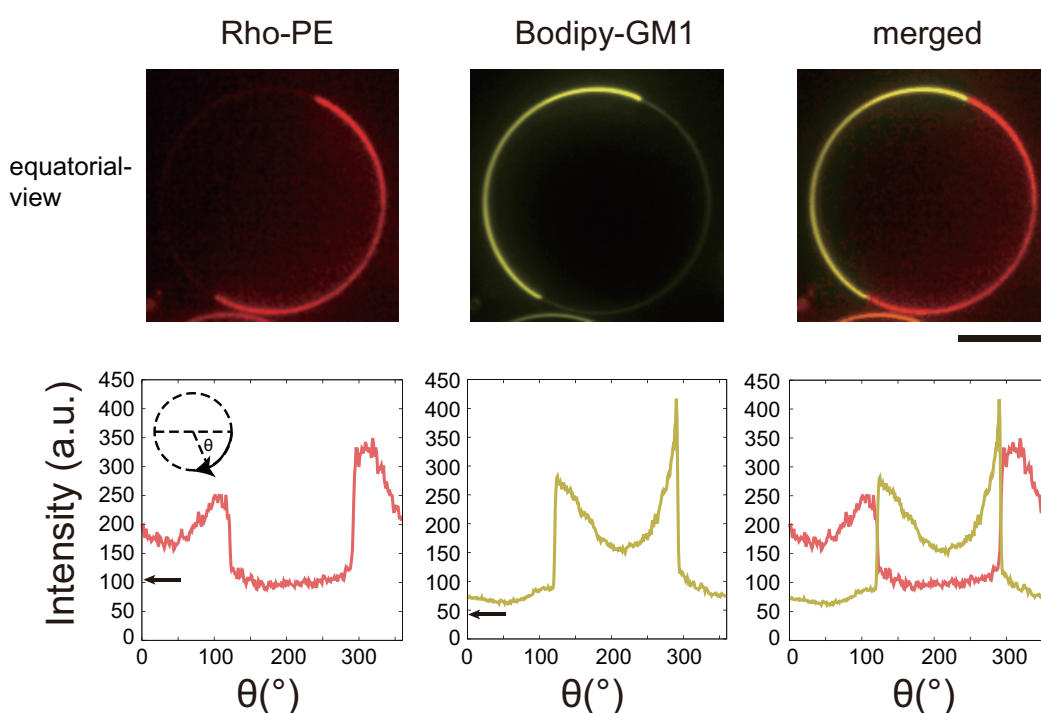


FIGURE 3.11: **Fluorescent imaging of GUVs after adding GM1.** Confocal cross-sectional views of a GUV. Rho-PE (0.8 mol %) and Bodipy-GM1 (1 mol %) stain L_d phase and GM1, respectively. The intensity profiles as a function of the angle θ are shown in the lower figures. The black arrows show background intensities. The scale bar is 10 μm . Added GM1 concentration is 250 μM . The figures were taken after about two hours of GM1 addition. The experiments were done at room temperature.

we calculated the average intensity of Bodipy-GM1 in each phase as a function of time after adding GM1. The time variation data in the L_o and L_d phases can be fitted by a single-exponential function with the characteristic time τ_1 , respectively (FIG. 3.12(a)). The characteristic time τ_1 in each phase is plotted as a function of added GM1 concentration (FIG. 3.12(b)). When adding 10 μM GM1, the characteristic time τ_1 in the L_o and L_d phases are 79.2 ± 17.7 and 23.3 ± 1.73 min, respectively.

When adding 100 μM GM1, the characteristic time τ_1 in the L_o and L_d phases are 6.2 ± 1.8 and 10.5 ± 3.3 min, respectively. When adding 250 μM GM1, The characteristic time τ_1 in the L_o and L_d phases are 3.9 ± 2.1 and 2.8 ± 1.1 min, respectively.

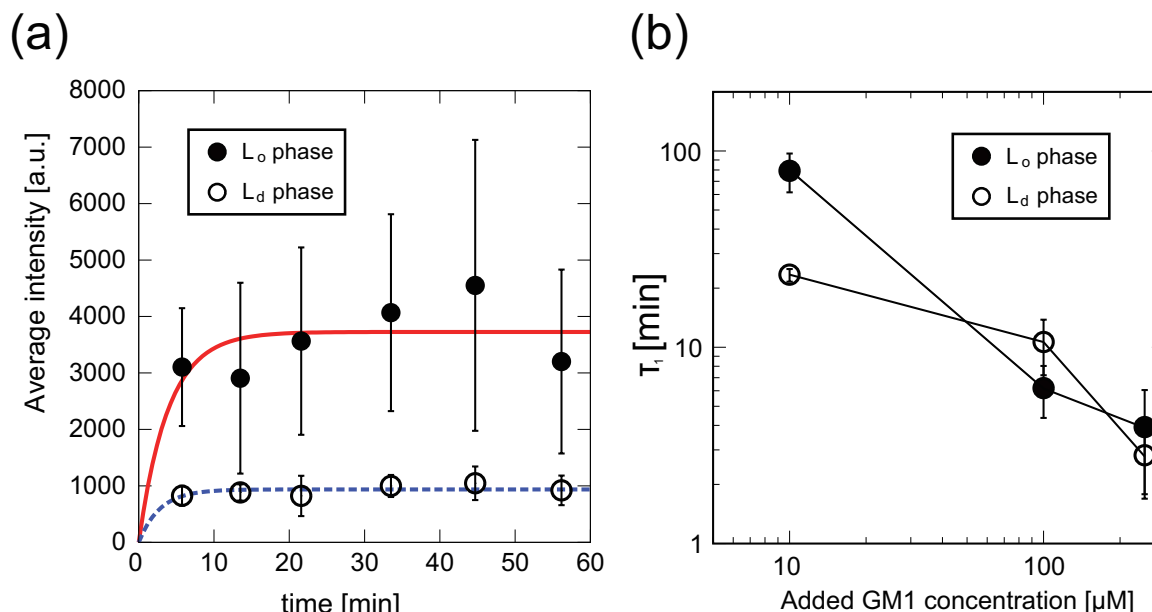


FIGURE 3.12: **Insertion dynamics of GM1 into lipid bilayers.** (a) The average intensities in L_o phase (closed circles) and L_d phase (open circles) are shown as a function of time. Added GM1 concentration is 250 μM . The time variation data in each phase can be fitted by a single-exponential function with the characteristic time τ_1 , respectively. The red-solid and blue-dashed lines have τ_1 of 3.9 ± 2.1 min and 2.8 ± 1.1 min, respectively. (b) Characteristic time τ_1 in L_o (closed circles) and L_d (open circles) phases as the functions of added GM1 concentration. The dots in each phase are connected by the hand-drawn straight line. The mole ratio between ternary components constituting GUVs was fixed at DOPC/DPPC/Chol=35/35/30. The experiments were performed at room temperature.

Quantitative estimation of the mole fractions of inserted GM1.

We quantitatively estimated the mole fraction of GM1 inserted in lipid bilayers after equilibrium. As the characteristic time of the insertion process is at most 100 min (FIG. 3.12(b)), after at least two hours of GM1 addition the intensity measurement was performed. The measured average intensities of Bodipy-GM1 (10 mol % for 10 μM and 50 μM , and 1 mol % for 75 μM , 90 μM , 100 μM , 200 μM and 250 μM) were compared with those in GUVs produced by the hydration of DOPC lipid film, including a well-defined quantity of Bodipy-GM1 (0.022, 0.056 and 0.120 mol %) (FIG. 3.13). Note that the average intensities were averaged along the equatorial orbits and then averaged using at least ten vesicles. From the linear fitting of the three data points, we obtained the relationship between the Bodipy-GM1 intensity

and the GM1 mole fraction, as shown by the black solid line (FIG. 3.13(b)). The solid line has slope $6.96 \times 10^4 \pm 3.97 \times 10^3$. Using the average intensities (FIG. 3.13(a)) and this linear relation (FIG. 3.13(b)), we quantitatively estimated the mole fraction of GM1 inserted in lipid bilayers from outside after equilibrium is reached.

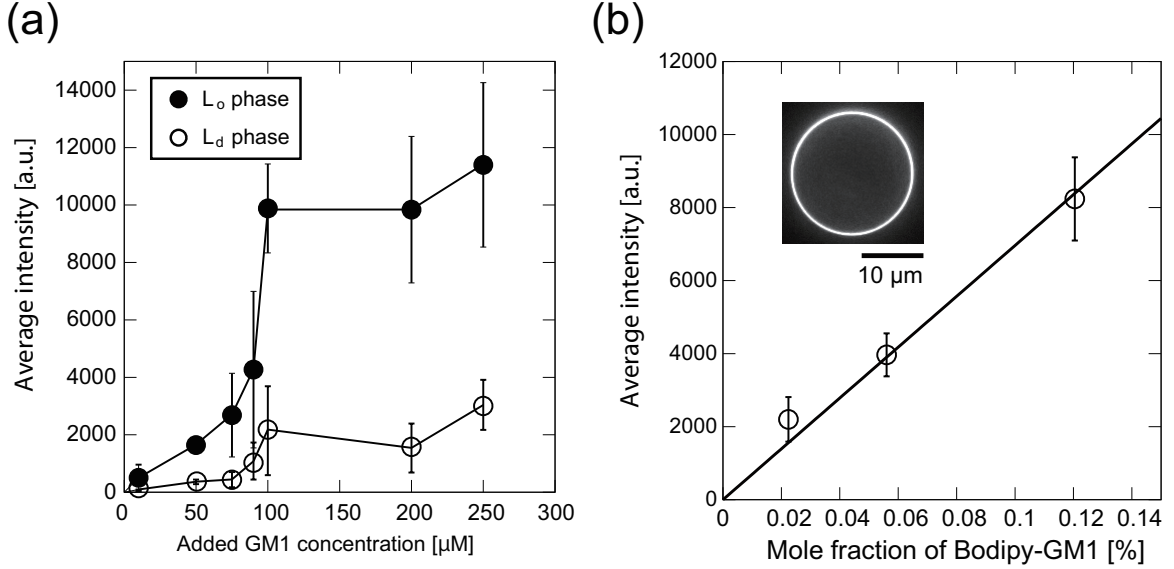


FIGURE 3.13: **Quantitative estimation of the mole fractions of inserted GM1.** (a) The average intensities of Bodipy-GM1 (10 mol % for 10 μM and 50 μM, and 1 mol % for 75 μM, 90 μM, 100 μM, 200 μM and 250 μM) in L_o (closed circles) and L_d (open circles) phase are shown as a function of the added GM1 concentration. The values of one-tenth of the measured ones for 10 μM and 50 μM are plotted. The mole ratio between ternary components constituting GUVs was fixed at DOPC/DPPC/Chol=35/35/30. (b) The average intensity of Bodipy-GM1 is shown as a function of the mole fraction of Bodipy-GM1 in DOPC GUVs. The solid line has slope $6.96 \times 10^4 \pm 3.97 \times 10^3$. The experiments were performed at room temperature.

Two coupled differential equations describing the successive transition

The phenomenological reaction rate equations for the macro-phase, $x_M(t)$, the stripe-phase, $x_s(t)$, and the micro-phase, $x_m(t) = 1 - x_M(t) - x_s(t)$ can be written as;

$$\frac{dx_M}{dt} = -k_1 x_M + k_2 x_s, \quad (3.2)$$

$$\frac{dx_s}{dt} = k_1 x_M - k_2 x_s - k_3 x_s + k_4 (1 - x_M - x_s), \quad (3.3)$$

where k_1 , k_2 , k_3 , and k_4 show the transition rates from the macro-phase to the stripe-phase, vice versa, from the stripe-phase to the micro-phase, and vice versa, respectively. The experimental observations allow us to assume the relationship $k_4 \simeq 0$

as we did not observe the transition from micro-phase to stripe-phase. Thus, the following equation is derived;

$$\frac{d}{dt} \begin{pmatrix} x_M \\ x_s \end{pmatrix} = \begin{pmatrix} -k_1 & k_2 \\ k_1 & -(k_2 + k_3) \end{pmatrix} \begin{pmatrix} x_M \\ x_s \end{pmatrix}. \quad (3.4)$$

Under the following boundary conditions, $x_M(0) = 1$, $x_M(\infty) = 0$, $x_s(0) = 0$ and $x_s(\infty) = 0$, $x_M(t)$, $x_s(t)$ and $x_m(t)$ are obtained as;

$$x_M(t) = e^{-\Lambda t} \left[\cosh(\sqrt{D}t) + \frac{\Lambda - k_1}{\sqrt{D}} \sinh(\sqrt{D}t) \right], \quad (3.5)$$

$$x_s(t) = \frac{k_1}{\sqrt{D}} e^{-\Lambda t} \sinh(\sqrt{D}t), \quad (3.6)$$

$$x_m(t) = 1 - e^{-\Lambda t} \left[\cosh(\sqrt{D}t) + \frac{\Lambda - 2k_1}{\sqrt{D}} \sinh(\sqrt{D}t) \right], \quad (3.7)$$

where $\Lambda = (k_1 + k_2 + k_3)/2$ and $D = \Lambda^2 - k_1 k_3$. the best-fit parameters, $k_1 = 0.670$, $k_2 = 0.173$ and $k_3 = 0.174$, are obtained by fitting the experimental results to the above theoretical functions using the Levenberg-Marquardt method.

Chapter 4

Numerical analysis of phase separation transitions from macro- to micro-phase separation

4.1 Introduction

In the previous chapter, we showed the experimental results of the novel transitions from macro- to micro-phase via a metastable stripe-phase in multi-component giant unilamellar vesicles (GUVs) with the asymmetric lipid composition. Considering the free energy functional form to describe phase separation coupled with shape deformation of lipid vesicles (see Eqs.(4.1) and (4.2)), the spontaneous curvatures and bending coefficients could be selected as the candidates to compete with the line energy and to drive the transitions, as discussed in the discussion of the previous chapter. However, the critical factor to drive the transitions has not been revealed yet. In this chapter, to reveal the factor, we numerically investigate the transitions using the time-dependent Ginzburg-Landau model, which describes phase separation and bending elastic membrane.

4.2 Theoretical analysis

In this section, we introduce the theoretical analysis for subsequent numerical simulations. The vesicle is represented by a two-dimensional closed surface parameterized by $u = (u^1, u^2)$. The order parameter ϕ is the local difference between the concentrations of DPPC (ϕ_{DPPC}) and DOPC (ϕ_{DOPC}) per unit area, i.e., $\phi = \phi_{\text{DPPC}} - \phi_{\text{DOPC}}$. We neglect GM1 diffusion because the GM1 was not rearranged after its initial insertion into L_o and L_d domains. We also ignore the temporal change of the asymmetric lipid composition under the flip-flop phenomena as it is recently reported that the lipid exchange of POPC (16:0/18:1), having acryl-chain length close to those of GM1 (16:0/18:1), DPPC (16:0/16:0) and DOPC (18:1/18:1), is quite slow (occurring with a half-live of 90 hours) [81]. In terms of ϕ , the free energy functional of the two-component vesicle $F = F_1 + F_2$ is expressed as

$$F_1 = \int \frac{\kappa(\phi)}{2} (H - H_{\text{sp}}(\phi))^2 \sqrt{g} d^2u + pV, \quad (4.1)$$

$$F_2 = \frac{k_B T}{a^2} \int \left[\frac{\xi_0^2}{2} (\nabla \phi)^2 + f(\phi) \right] \sqrt{g} d^2 u, \quad (4.2)$$

where F_1 is the sum of the bending elastic and osmotic energies. In this expression, $\sqrt{g} d^2 u$ is the area element and $\kappa(\phi)$ denotes the bending elastic modulus. $H/2$ and $H_{\text{sp}}(\phi)$ are the mean and spontaneous curvature, respectively; p is the osmotic pressure difference across the vesicle, and V is the enclosed volume. The composition-dependent Gaussian curvature term is excluded because its magnitude has not been experimentally clarified yet. The surface tension term is also ignored because of the incompressibility condition. The following simple functional forms are assumed for $\kappa(\phi)$ and $H_{\text{sp}}(\phi)$:

$$\kappa(\phi) = \kappa_0 + \kappa_1 \phi, \quad (4.3)$$

$$H_{\text{sp}}(\phi) = H_{\text{sp}}^{(0)} + H_{\text{sp}}^{(1)} \phi \quad (4.4)$$

where κ_0 , κ_1 , $H_{\text{sp}}^{(0)}$ and $H_{\text{sp}}^{(1)}$ are constants. F_2 is the Ginzburg-Landau free energy at temperature T , where k_B is the Boltzmann constant, a denotes the unit cell length, and ξ_0 is the correlation length. The potential $f(\phi)$ is written by:

$$f(\phi) = a_2 \phi^2 / 2 + a_4 \phi^4 / 4, \quad (4.5)$$

where a_2 is expressed as $|a|(T - T_c)$, with the critical temperature T_c and a_4 are the positive coefficient. Considering the situation $T > T_c$, a_2 is the negative coefficient.

We now consider the time evolution of the phase-separation transitions. Denoting the position vector of a material point u at time t by $\mathbf{r}(u, t)$, the simplest dissipative model is

$$\frac{\partial \mathbf{r}(u, t)}{\partial t} = -\Gamma \frac{\delta}{\delta \mathbf{r}(u, t)} \left[F + \int \gamma(u, t) \sqrt{g} d^2 u \right], \quad (4.6)$$

where Γ is a kinetic coefficient. Here, the local Lagrange multiplier $\gamma(u, t)$ imposes the local incompressibility condition [19, 20]. From Eqs. (4.1)-(4.6), the equation of motion is explicitly derived as

$$\frac{\partial \mathbf{r}(u, t)}{\partial t} = -\Gamma [A_{\perp} \mathbf{n} + A_{\alpha} \mathbf{g}^{\alpha}], \quad (4.7)$$

where $A_{\perp}$ and A_{α} are the normal and tangential forces to the surface along a curvilinear coordinate u^{α} , respectively, and $\mathbf{n}$ and $\mathbf{g}^{\alpha}$ are the normal and tangential unit vectors, respectively. We investigate the dynamics of a 2D vesicle (equivalent to an infinitely long cylinder) and a spherical 3D vesicle. We now describe the details of the equations for the spherical 3D vesicle [19, 20]. See supplementary information for further details of the equations for the spherical 2D vesicle (see supplementary information: "Dynamics of x , y , γ , and ϕ in a two-dimensional vesicle"). The normal

force to the surface $A_\perp$ and tangential force A_α are expressed as;

$$A_\perp = -\xi^2 b^{\alpha\beta} \phi_{,\alpha} \phi_{,\beta} + P - B(H^2 - 2K) - \Delta_{\text{LB}} B + \bar{\gamma} H \quad (4.8)$$

$$A_\alpha = \xi^2 (\phi_{,\alpha} \phi_{,\beta})|^\beta + B H_{,\alpha} - \bar{\gamma}_{,\alpha}, \quad (4.9)$$

where

$$B = \kappa(\phi)[H - H_{\text{sp}}(\phi)], \quad (4.10)$$

and

$$\bar{\gamma} = \gamma + \frac{\kappa H^2}{2} + [f(\phi) + \frac{\xi^2}{2}(\nabla\phi^2)]. \quad (4.11)$$

The local incompressibility condition, $\partial\sqrt{g}/\partial t = 0$, gives us the equation for $\bar{\gamma}$;

$$(\Delta_{\text{LB}} - H^2)\bar{\gamma} - C = 0, \quad (4.12)$$

where C is defined as;

$$C = -H[\xi^2 b^{\alpha\beta} \phi_{,\alpha} \phi_{,\beta} + B(H^2 - 2K) + \Delta_{\text{LB}} B] \\ - P + \xi^2 (\phi_{,\alpha} \phi_{,\beta})|_{\alpha\beta} + (A H_{,\alpha})|_\alpha \quad (4.13)$$

Next, we consider phase separation in the lipid bilayers. For simplicity, the contribution of the hydrodynamic flow to phase separation dynamics is ignored. The temporal dynamics of the conserved order parameter $\phi(u, t)$ follow the continuity equation;

$$\frac{\partial\phi(u, t)}{\partial t} = -J_\alpha|^\alpha. \quad (4.14)$$

See Chapter 2 for the definition of the operator $|^\alpha$. The total flux J_α is the sum of thermodynamic fluxes induced by the chemical potential gradients $\mu(u, t)$ and the random current, $J_\alpha^{(r)}$, which satisfies the fluctuation dissipation theorem [20]. Specifically, we have

$$J_\alpha = -L \frac{\partial\mu}{\partial u^\alpha} + J_\alpha^{(r)} \quad (4.15)$$

where L is the transport coefficient. The random current satisfies the following dissipation theorem;

$$< J_\alpha^{(r)}(u, t) J_\beta^{(r)}(u', t') > = \\ 2k_B T L \delta_{\alpha\beta} \frac{1}{\sqrt{g}} \delta(u^1 - u'^1) \delta(u^2 - u'^2) \delta(t - t') \quad (4.16)$$

where $< \dots >$ denotes the statistical average of $(\dots)$. The chemical potential μ , expressed as $\delta F/\delta\phi$, is calculated as follows:

$$\mu = \frac{k_B T}{a^2} \left[-\xi_0^2 \Delta_{\text{LB}} \phi + f' \right] + \frac{1}{2} \kappa_1 (H - H_{\text{sp}})^2 - B H_{\text{sp}}^{(1)}, \quad (4.17)$$

where Δ_{LB} is the Laplace-Beltrami operator, and $f' = \partial f/\partial\phi$.

In our simulation, space, time, and energy were scaled by ξ , τ_0 , and ε_0 , respectively, where

$$\begin{aligned}\xi^2 &= \frac{\xi_0^2}{|a_2|}, \\ \tau_0 &= \frac{a^{d-1}\xi^2}{k_B T L |a_2|}, \\ \text{and } \varepsilon_0 &= \frac{|a_2|\phi_{\text{eq}}^2 k_B T \xi^2}{a^{d-1}},\end{aligned}\tag{4.18}$$

with dimension d and ϕ_{eq} defined as $\sqrt{|a_2|/a_4}$. This non-dimensionalization procedure yields two scaled bending coefficients,

$$\begin{aligned}\tilde{\kappa}_0 &= \frac{\kappa_0}{\varepsilon_0}, \\ \text{and } \tilde{\kappa}_1 &= \frac{\kappa_1 \phi_{\text{eq}}}{\kappa_0},\end{aligned}\tag{4.19}$$

two scaled spontaneous curvatures,

$$\begin{aligned}\tilde{H}_{\text{sp}}^{(0)} &= H_{\text{sp}}^{(0)} \xi, \\ \text{and } \tilde{H}_{\text{sp}}^{(1)} &= H_{\text{sp}}^{(1)} \phi_{\text{eq}} \xi,\end{aligned}\tag{4.20}$$

a scaled damping coefficient,

$$\tilde{\Gamma} = \frac{\Gamma \tau_0 \kappa_0}{\xi^4},\tag{4.21}$$

the strength of the random current,

$$\tilde{\eta} = \frac{\sqrt{2(a/\xi)^{d-1}/|a_2|}}{\phi_{\text{eq}}},\tag{4.22}$$

and time

$$\tilde{t} = \frac{t}{\tau_0}.\tag{4.23}$$

See supplementary information with regard to the non-dimensionalization of the random current.

4.3 Results

The radius and parameters (except for $\tilde{\kappa}_0$, $\tilde{\Gamma}$, $\tilde{\eta}$, and $\tilde{t}$) of our simulated 2D vesicles were those of the experimentally investigated vesicles. On the other hand, to reduce the computation cost, we shrank the system size of the simulated 3D vesicles by approximately 10 times and qualitatively compared the dynamics of the numerically and experimentally investigated vesicles. The initial radius of the 2D circular

vesicles was estimated as $N\xi/2\pi$, where N is the number of material points. For example, if $N = 2000$ and $\xi = 10$ nm (a reasonable assumption), the simulated vesicle radius is $3.2 \mu\text{m}$, close to the average vesicle radius observed in the experiments (FIG. 3.2). The bending coefficient $\tilde{\kappa}_0$ is the undetermined parameter because it is difficult to determine from the experimental values. The bending coefficient $\tilde{\kappa}_1$ was explicitly estimated from the ratio of the bending coefficients in the DPPC-rich phase and the DOPC-rich phase, using Eq. (4.3) and the reported value 1.25 [18], as follows:

$$\frac{\kappa_0 + \kappa_1\phi_{\text{eq}}}{\kappa_0 - \kappa_1\phi_{\text{eq}}} = 1.25, \quad (4.24)$$

leading to

$$\tilde{\kappa}_1 = 1/9. \quad (4.25)$$

The spontaneous curvature of the bilayer in each phase was then computed as

$$H_{\text{sp}} = \frac{H_{\text{sp}}^{\text{out}} - H_{\text{sp}}^{\text{in}}}{2} \quad (4.26)$$

where $H_{\text{sp}}^{\text{in}}$ and $H_{\text{sp}}^{\text{out}}$ are the spontaneous curvatures of the inner and outer monolayers, respectively [82, 83]. The spontaneous curvature of a monolayer was assumed as the linear function of the mole fractions of the components [84, 85]. Using the reported spontaneous curvatures of DPPC, DOPC, and GM1 (0.03 nm^{-1} [86], -0.06 nm^{-1} [84] and 0.17 nm^{-1} [80], respectively) and the measured mole fractions of GM1 after adding $75 \mu\text{M}$ GM1 solution ($\sim 3.85\%$ in L_o phase and $\sim 0.63\%$ in L_d phase), we obtained the spontaneous curvatures of the bilayers in both phases (see supplementary information: "Quantitative estimation of the mole fractions of inserted GM1" in the previous chapter and FIG. 6.2(c)). Given the above values, $\tilde{H}_{\text{sp}}^{(0)}$ and $\tilde{H}_{\text{sp}}^{(1)}$ were estimated as 0.068 and 0.040, respectively. The values of $\tilde{\Gamma}$ in the 2D and 3D simulations were 1 and 0.1, respectively. The values of $\tilde{\eta}$ in the 2D and 3D simulations were 0 and 0.5, respectively.

In our simulation, the spontaneous curvature of the fully phase-separated vesicle before adding GM1 ($\tilde{t} < 0$) was set to zero, i.e., $\tilde{H}_{\text{sp}}^{(0)} = 0$ and $\tilde{H}_{\text{sp}}^{(1)} = 0$. The vesicle was sufficiently relaxed to an equilibrium state. At $\tilde{t} = 0$ we assumed that GM1 was instantly inserted into the outer layer, changing the spontaneous curvature in each domain. Setting the non-zero spontaneous curvatures, we observed the following process from $\tilde{t} = 0$ to $\tilde{t} = 500$ (in 2D) and $\tilde{t} = 8500$ (in 3D). Instantaneous insertion of GM1 is reasonable, as insertion occurs on a much shorter time scale than the subsequent phase separation processes. In the 2D vesicles, we observed the transitions from macro- to micro-phase separation with shape deformations (FIG. 4.1(a)). The parameters were set to $N = 2000$, $\tilde{\kappa}_0 = 300$, $\tilde{\kappa}_1 = 1/9$, $\tilde{H}_{\text{sp}}^{(0)} = 0.068$, $\tilde{H}_{\text{sp}}^{(1)} = 0.040$, $\tilde{\Gamma} = 1$, and $\tilde{\eta} = 0$. In the 3D vesicles, the transitions from macro- to micro-phase occurred via a stripe-micro coexistence state (FIG. 4.2). The parameters were set to $N = 20252$, $\tilde{\kappa}_0 = 100$, $\tilde{\kappa}_1 = 1/9$, $\tilde{H}_{\text{sp}}^{(0)} = 0.068 \times 2$ and $\tilde{H}_{\text{sp}}^{(1)} = 0.040 \times 2$, with $\tilde{\Gamma} = 0.1$ and $\tilde{\eta} = 0.5$. To balance the effects of spontaneous curvatures on the dynamics of the 2D and 3D vesicles, the spontaneous curvatures in the 3D simulations were doubled. In FIG. 4.1 and 4.2, green and red areas

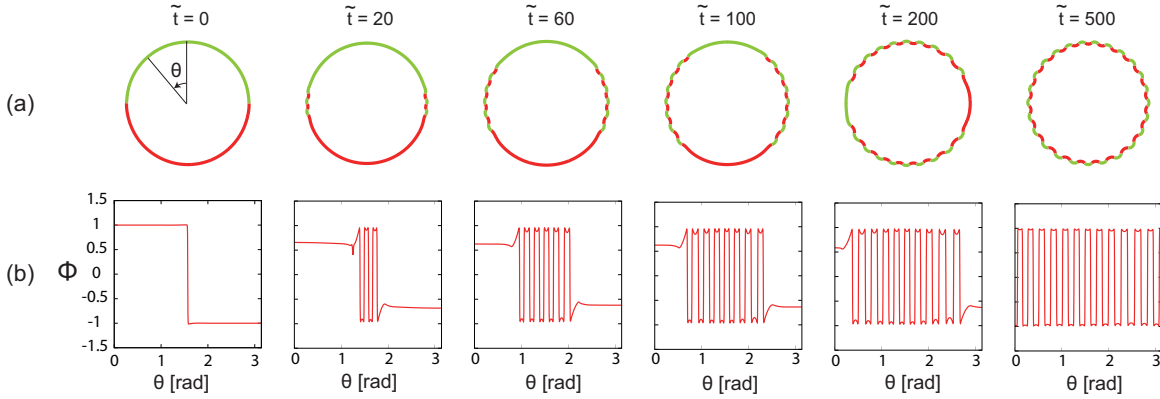


FIGURE 4.1: Transition dynamics from macro- to micro-phase separation induced by a sudden change of spontaneous curvature. (a) 2D vesicles at $\tilde{t}=0, 20, 60, 100, 200$ and 500 . The spontaneous curvatures were estimated from the measured mole fraction of GM1 ($\sim 3.85\%$ in L_o phase and $\sim 0.63\%$ in L_d phase) when $75 \mu\text{M}$ GM1 solution was added. (b) Profiles of ϕ as a function of the angle θ . The domains where $\phi \geq 0$ (rich in DPPC) and $\phi < 0$ (rich in DOPC) are shown in green and red, respectively. The parameters were set to $N = 2000$, $\tilde{\kappa}_0 = 300$, $\tilde{\kappa}_1 = 1/9$, $\tilde{H}_{\text{sp}}^{(0)} = 0.068$, $\tilde{H}_{\text{sp}}^{(1)} = 0.040$, $\tilde{\Gamma} = 1$, and $\tilde{\eta} = 0$.

represent the DPPC-rich ($\phi \geq 0$) and DOPC-rich ($\phi < 0$) domains, respectively.

4.4 Discussion

We now discuss the consistency between the experimental results (see the previous chapter) and numerical results. In both of the 2D and 3D numerical simulations, we found that the monodispersed L_o domains were distributed at approximately regular intervals and slightly budded toward the outside of the vesicle, surprisingly similar to the experimental results. In qualitative terms, successive transitions increase the boundary energy F_2 , but decrease the bending elastic energy F_1 , because the membrane and spontaneous curvature approach under the bending of the L_o phase. This activity decreases the total free energy. Note that the bending energy is decreased more by fitting the curvature of the stiffer L_o domains to a local spontaneous curvature. The distribution of the L_o domains at approximately regular intervals might be ascribed to the long-range repulsion derived from the bending energy of the L_d matrix between two adjacent L_o domains, as suggested by past papers [21, 87]. Moreover, in the simulated 3D vesicles, the quenched macro-phase transits to the micro-phase not through the stripe-state but through a strip-micro state. This transition, which differs from the experimental results as shown in the previous chapter, can be attributed to a stable or metastable stripe-micro coexisting state, which emerges between the macro- and micro-states as a function of spontaneous curvature under the parameter set (FIG. 4.3). The non-occurrence of a true stripe-phase in the numerical analysis warrants further theoretical investigation.

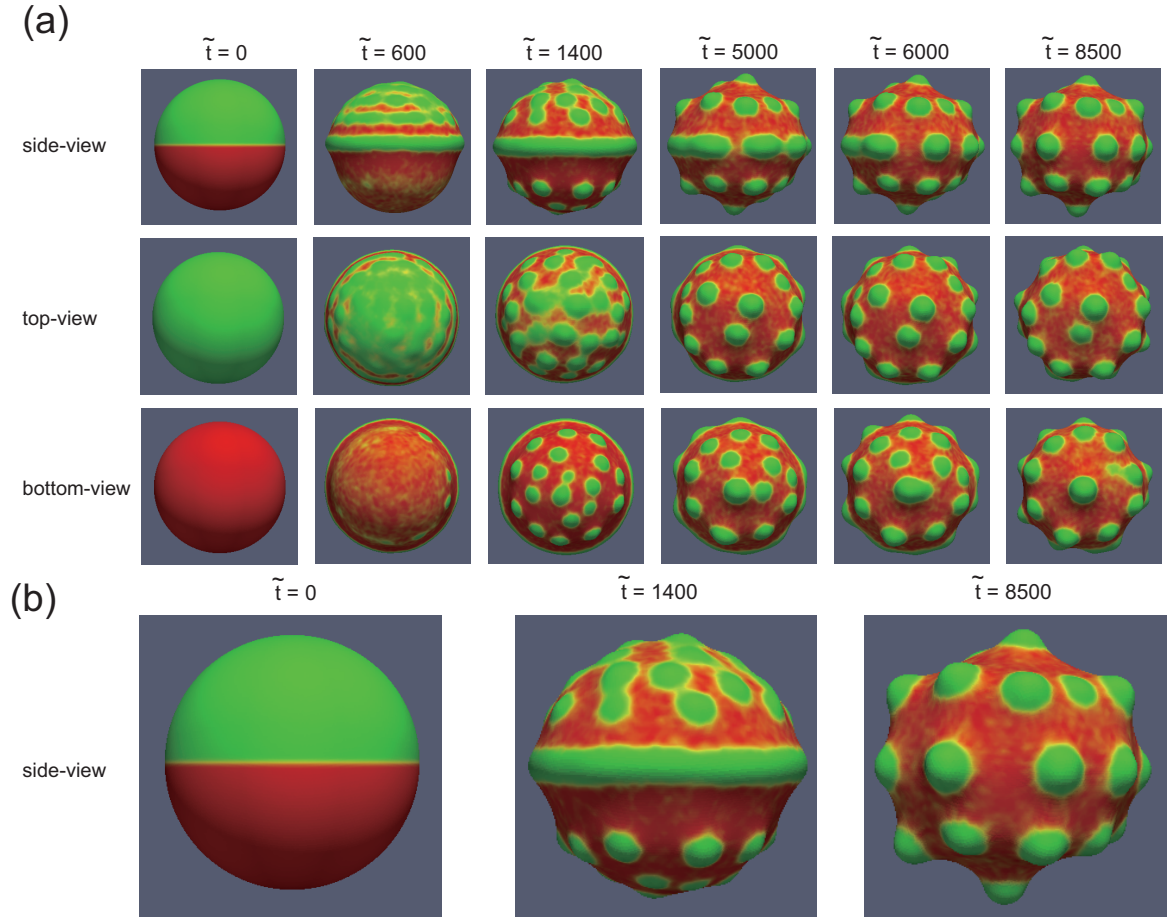


FIGURE 4.2: Transition dynamics from macro- to micro-phase separation induced by a sudden change of spontaneous curvature. (a) Side, top, and bottom-views of 3D vesicles at $\tilde{t}=0, 600, 1400, 5000, 6000$ and 8500 . (b) Enlarged side-views of 3D vesicles at $\tilde{t}=0, 1400$, and 8500 . The domains where $\phi \geq 0$ (rich in DPPC) and $\phi < 0$ (rich in DOPC) are shown in green and red, respectively. The spontaneous curvatures were estimated from the measured mole fraction of GM1 ($\sim 3.85\%$ in L_o phase and $\sim 0.63\%$ in L_d phase) when $75 \mu\text{M}$ GM1 solution was added. The parameters were set to $N = 20252$, $\tilde{\kappa}_0 = 100$, $\tilde{\kappa}_1 = 1/9$, $\tilde{H}_{\text{sp}}^{(0)} = 0.068 \times 2$ and $\tilde{H}_{\text{sp}}^{(1)} = 0.040 \times 2$, with $\tilde{\Gamma} = 0.1$ and $\tilde{\eta} = 0.5$.

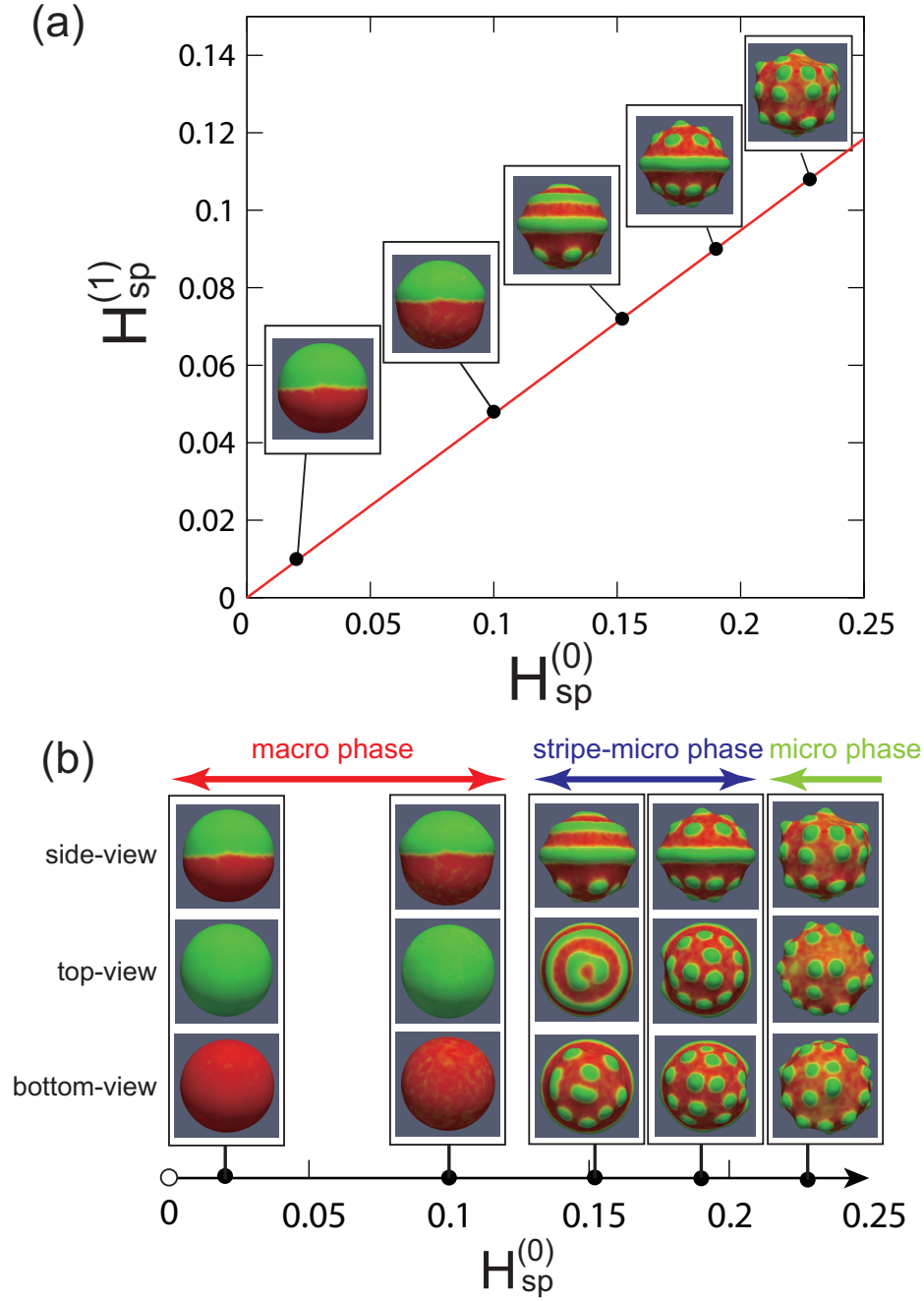


FIGURE 4.3: **Emergence of stripe-micro phase between macro- and micro-phase as a function of spontaneous curvatures.** (a) The solid circles represent the values of a pair of spontaneous curvatures, $\tilde{H}_{sp}^{(0)}$ and $\tilde{H}_{sp}^{(1)}$, used in the numerical simulation. The red-solid line has slope 0.47, which was estimated from the linear relation between the mole fraction of GM1 in L_o and L_d phases, which was measured in the experiments (FIG. 4(c)). The inset images show 3D vesicles at $t = 1000$ after setting the positive curvatures. See below for the details for the parameter values. (b) The side, top and bottom views of 3D vesicles at $t = 1000$ are shown as a function of $\tilde{H}_{sp}^{(0)}$. The used parameters are as follows: $N = 9002$, $\tilde{\kappa}_0 = 300$, $\tilde{\kappa}_1 = 1/9$, $\tilde{\Gamma} = 0.1$ and $(\tilde{H}_{sp}^{(0)}, \tilde{H}_{sp}^{(1)}) = (0.020, 0.010), (0.100, 0.048), (0.152, 0.072), (0.190, 0.090)$ and $(0.228, 0.108)$. The coexisting phase of stripe- and micro-phase are observed between macro- and micro-phase.

Next, we discuss the consistency between the experimental and numerical results on the stability of each phase in the transitions. The time scale of the whole transitions from the macro- to micro-phase in the experiments is much larger than that of each transition, as mentioned above. These experimental results imply that, in our experimental time scale, the quenched macro- and stripe-phases are metastable, and the micro-phase is stable. On the other hand, according to the numerical results, the stripe-micro and micro-phases are metastable and stable in our numerical time scale, respectively, which is consistent with the experimental results. However, numerical results tell us that the stability of the quenched macro-phase at the initial state seems to be unstable, which may be not consistent between the experimental and numerical results. In fact, the stability of the quenched macro-phase at the initial state in the numerical simulations is determined by the magnitude of tuned spontaneous curvature. At a critical magnitude, the stability of the quenched macro-phase should change from a stable or metastable state to an unstable state. Further numerical investigation would be required for revealing whether the metastable quenched macro-phase transits to stable micro-phase by tuning the parameter values and/or using another model, e.g. model H.

4.5 Summary

To summarize, by numerically analyzing the bending elastic model and the time-dependent Ginzburg-Landau equation, we gained insights into the mechanism of the novel transitions, which is apparently governed by the local spontaneous curvature induced by the local asymmetric composition due to externally added glycolipids (GM1).

4.6 Supplementary information

Dynamics of x , y , γ , and ϕ in a two-dimensional vesicle

The two-dimensional vesicle is represented by a one-dimensional closed line, parameterized by s . The position vector of a material point s at time t is denoted by $\mathbf{r}(s, t) = (x(s, t), y(s, t))$. The differential equations governing $x(s, t)$ and $y(s, t)$ are given by

$$\begin{aligned} \frac{\partial x(s, t)}{\partial t} = -\Gamma & \left[-\frac{\partial^2}{\partial s^2} \left(B \frac{\partial y}{\partial s} \right) - \frac{\partial}{\partial s} \left(B \frac{\partial^2 y}{\partial s^2} \right) + p \frac{\partial y}{\partial s} \right. \\ & \left. - \frac{\partial}{\partial s} \left(\gamma(s, t) \frac{\partial x}{\partial s} \frac{1}{\sqrt{(\frac{\partial x}{\partial s})^2 + (\frac{\partial y}{\partial s})^2}} \right) \right], \end{aligned} \quad (4.27)$$

$$\begin{aligned} \frac{\partial y(s, t)}{\partial t} = -\Gamma \left[\frac{\partial^2}{\partial s^2} \left(B \frac{\partial x}{\partial s} \right) + \frac{\partial}{\partial s} \left(B \frac{\partial^2 x}{\partial s^2} \right) - p \frac{\partial x}{\partial s} \right. \\ \left. - \frac{\partial}{\partial s} \left(\gamma(s, t) \frac{\partial y}{\partial s} \frac{1}{\sqrt{(\frac{\partial x}{\partial s})^2 + (\frac{\partial y}{\partial s})^2}} \right) \right], \end{aligned} \quad (4.28)$$

where B is defined as $\kappa(\phi)[H - H_{\text{sp}}(\phi)]$.

To derive the governing equation for $\gamma(s, t)$, we substitute the differential equations of x and y into the relation:

$$\partial g / \partial t = 0, \quad (4.29)$$

where

$$g = \left(\frac{dx}{ds} \right)^2 + \left(\frac{dy}{ds} \right)^2 = 1, \quad (4.30)$$

leading to

$$\ddot{\gamma} - H^2 \gamma - D = 0, \quad (4.31)$$

where

$$D = pH - \dot{x}\ddot{A}_y + \dot{y}\ddot{A}_x, \quad (4.32)$$

$$A_\alpha \equiv \dot{B}\dot{\alpha} + 2B\ddot{\alpha} \quad (\alpha \in \{x, y\}), \quad (4.33)$$

and the dot above a variable X denotes $\partial X / \partial s$.

The temporal dynamics of the conserved parameter $\phi(s, t)$ are given by the continuity equation,

$$\frac{\partial \phi(s, t)}{\partial t} = -\frac{\partial}{\partial s} J(s, t). \quad (4.34)$$

The diffusion flux J is proportional to the lateral gradient of the local chemical potential $\mu(s, t)$, namely

$$J = -L \frac{\partial \mu(s, t)}{\partial s}, \quad (4.35)$$

where L is the transport coefficient. The chemical potential μ , given by $\delta F / \delta \phi(s, t)$, is calculated as follows:

$$\mu(s, t) = \frac{k_B T}{a} \left[-\xi_0^2 \frac{\partial^2 \phi}{\partial s^2} + \frac{\partial f}{\partial \phi} \right] + \frac{1}{2} \kappa_1 (H - H_{\text{sp}})^2 - B H_{\text{sp}}^{(1)} \quad (4.36)$$

Therefore, we can investigate phase separation coupled with shape deformation by solving the coupled differential equations for x , y , γ , and ϕ .

Non-dimensionalization of the random current $J^{(r)}$

We describe the non-dimensionalization of the random current $\vec{J}^{(r)}(u, v)$ on an arbitrary curved 2D surface expressed by (u, v) . The random current $\vec{J}^{(r)}(u, v)$ represents the collisions with the molecules, considered to be the white-Gaussian noise and the stochastic process. Thus, its statistical properties such as the average and standard deviation are included in the simulation. The continuity equation is given

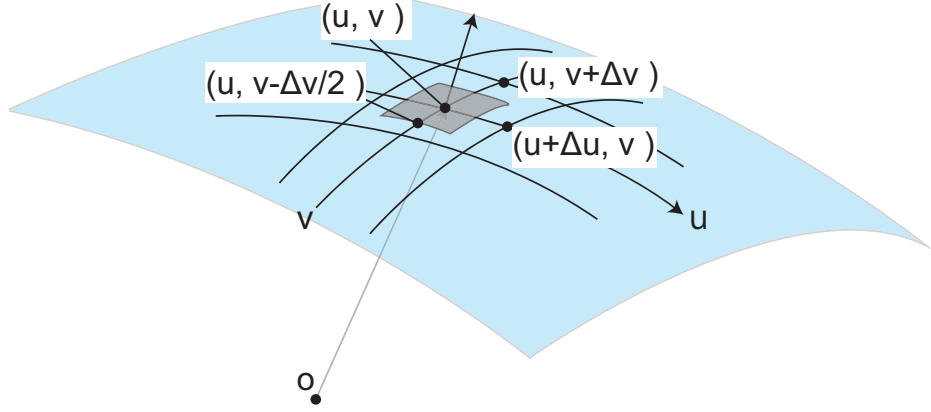


FIGURE 4.4: Schematic of an arbitrary curved surface parameterized by (u, v) .

by:

$$\frac{\partial \tilde{\phi}}{\partial \tilde{t}} = \frac{\partial^2 \tilde{\mu}}{\partial \tilde{u}^2} + \frac{\partial^2 \tilde{\mu}}{\partial \tilde{v}^2} - \frac{\tau_0}{\xi \phi_{\text{eq}}} \left[\frac{\partial J_u^{(r)}}{\partial \tilde{u}} + \frac{\partial J_v^{(r)}}{\partial \tilde{v}} \right], \quad (4.37)$$

where $J_u^{(r)}$ and $J_v^{(r)}$ are the u and v components of $\vec{J}^{(r)}(u, v)$. We can extract the statistical properties of the random current $\vec{J}^{(r)}(u, v)$ by integrating Eq. (4.37) from $\tilde{t}$ to $\tilde{t} + \Delta \tilde{t}$, and integrating the third term on the right side from $\tilde{X} - \Delta \tilde{X}/2$ to $\tilde{X} + \Delta \tilde{X}/2$ ($X \in \{u, v\}$) and dividing by $\Delta \tilde{u} \Delta \tilde{v}$;

$$\begin{aligned} \tilde{\phi}(\tilde{t} + \Delta \tilde{t}) &= \tilde{\phi}(\tilde{t}) + \Delta \tilde{t} \left[\frac{\partial^2 \tilde{\mu}}{\partial \tilde{u}^2} + \frac{\partial^2 \tilde{\mu}}{\partial \tilde{v}^2} \right] \\ &\quad - \frac{\tau_0}{\xi \phi_{\text{eq}} \Delta \tilde{u} \Delta \tilde{v}} \int_{\tilde{t}}^{\tilde{t} + \Delta \tilde{t}} \int_{\tilde{u} - \Delta \tilde{u}/2}^{\tilde{u} + \Delta \tilde{u}/2} \int_{\tilde{v} - \Delta \tilde{v}/2}^{\tilde{v} + \Delta \tilde{v}/2} \left[\frac{\partial J_u^{(r)}}{\partial \tilde{u}} + \frac{\partial J_v^{(r)}}{\partial \tilde{v}} \right] d\tilde{u} d\tilde{v} d\tilde{t} \\ &= \tilde{\phi}(\tilde{t}) + \Delta \tilde{t} \left[\frac{\partial^2 \tilde{\mu}}{\partial \tilde{u}^2} + \frac{\partial^2 \tilde{\mu}}{\partial \tilde{v}^2} \right] \\ &\quad - \frac{\tau_0}{\xi \phi_{\text{eq}} \Delta \tilde{u} \Delta \tilde{v}} \int \int \int \left[\frac{J_u^{(r)}(u + \Delta u/2, v, t) - J_u^{(r)}(u - \Delta u/2, v, t)}{\Delta \tilde{u}} + \frac{\partial J_v^{(r)}}{\partial \tilde{v}} \right] d\tilde{u} d\tilde{v} d\tilde{t}. \end{aligned} \quad (4.38)$$

We now focus on a part of the above right-side, denoted by A ;

$$A = \frac{1}{(\Delta u^2 \Delta v)} \int_{\tilde{t}}^{\tilde{t} + \Delta \tilde{t}} \int_{\tilde{u} - \Delta \tilde{u}/2}^{\tilde{u} + \Delta \tilde{u}/2} \int_{\tilde{v} - \Delta \tilde{v}/2}^{\tilde{v} + \Delta \tilde{v}/2} J_u^{(r)}\left(u + \frac{\Delta u}{2}, v, t\right) d\tilde{u} d\tilde{v} d\tilde{t}. \quad (4.39)$$

The average of A is zero and the standard deviation σ^2 is calculated as;

$$\begin{aligned}
\sigma^2 &= \frac{1}{(\Delta u^2 \Delta v)^2} \int \int dt dt' \int \int du du' \int \int dv dv' < J_u^{(r)}(u + \frac{\Delta u}{2}, v, t) J_u^{(r)}(u' + \frac{\Delta u}{2}, v', t') > \\
&= \frac{2k_B T L}{(\Delta u^2 \Delta v)^2} \int \int dt dt' \int \int du du' \int \int dv dv' \delta(u - u') \delta(v - v') \delta(t - t') / \sqrt{g} \\
&= \frac{\Delta t \Delta u \Delta v 2k_B T L}{(\Delta u^2 \Delta v)^2 \tau \xi^2 \sqrt{g}},
\end{aligned} \tag{4.40}$$

where we used the fluctuation dissipation theorem. Calculating the other terms on the right-side in Eq. (4.38) as well, the equation can be described by:

$$\begin{aligned}
\tilde{\phi}(\tilde{t} + \Delta \tilde{t}) &= \tilde{\phi}(\tilde{t}) + \Delta \tilde{t} \left[\frac{\partial^2 \tilde{\mu}}{\partial \tilde{u}^2} + \frac{\partial^2 \tilde{\mu}}{\partial \tilde{v}^2} \right] - \tilde{R} \cdot \sqrt{\Delta \tilde{t}} \\
&\quad \left[[w(\tilde{u} + \Delta \tilde{u}/2, \tilde{v}, \tilde{t}) - w(\tilde{u} - \Delta \tilde{u}/2, \tilde{v}, \tilde{t})] \cdot \Delta \tilde{l}_u \right. \\
&\quad \left. + [w(\tilde{u}, \tilde{v} + \Delta \tilde{v}/2, \tilde{t}) - w(\tilde{u}, \tilde{v} - \Delta \tilde{v}/2, \tilde{t})] \cdot \Delta \tilde{l}_v \right],
\end{aligned} \tag{4.41}$$

where

$$\tilde{R} = \frac{\tau}{\xi \phi_{\text{eq}}} \sqrt{\frac{2k_B T L}{\tau \xi^2 \sqrt{g}}}, \tag{4.42}$$

representing the strength of the random current, w is the standard normal random number, and $\tilde{l}_u$ and $\tilde{l}_v$ are expressed as;

$$\begin{aligned}
\tilde{l}_u &= \frac{1}{(\Delta \tilde{u}^3 \Delta \tilde{v})} \\
\tilde{l}_v &= \frac{1}{(\Delta \tilde{u} \Delta \tilde{v}^3)}.
\end{aligned} \tag{4.43}$$

The standard normal random number was produced using by the Box-Muller method.

Chapter 5

The effect of GM1 on the bending modulus of lipid bilayers

5.1 Introduction

In the previous chapter, we showed the numerical results for investigating the essential factors of the transitions from macro- to micro-phase separation in multi-component GUVs, based on the purely dissipative model to describe bending elastic membrane and phase separation. The numerical simulations gave us insights into the mechanism, which is apparently governed by the local spontaneous curvature induced by the local asymmetric lipid composition. In the framework, we have made some assumptions in the simulations for simplicity. One of the most important assumptions is that the ratio of the bending moduli in the L_o and L_d phases is not affected by GM1 inserted into lipid bilayers. In this chapter, to clarify whether this assumption is valid or not, we reveal the effects of GM1 inserted into the L_o and L_d phases on the bending moduli by analyzing membrane fluctuations.

5.2 Materials and methods

Materials

DOPC (Dioleoylphosphatidylcholine), DPPC (Dipalmitoylphosphatidylcholine), Cholesterol, and GM1 (monosialotetrahexosylganglioside) were purchased from Avanti Polar Lipids (Alabaster, AL). Texas Red-DHPE (1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt) were purchased from Molecular Probes. DOPC, DPPC, and Texas Red-DHPE were dissolved in chloroform and stored at -20°C in a freezer. Cholesterol was dissolved in chloroform with methanol (50 % w/w) and stored at -20°C in a freezer. GM1 was dissolved in 200 mM glucose. After ultrasonication for 90 minutes 55°C , GM1 solution was left at least one hour before the use. It was stored at 4°C in a fridge. Sucrose and glucose were purchased from Nacalai Tesque, dissolved in Milli-Q water, and filtered through a Millipore syringe filter (Millex-GS, $0.22\text{ }\mu\text{m}$ pore size).

GUV preparation

GUVs were prepared by electroformation. DOPC (3.57 mM, 150 μ L) or DPPC (3.57 mM, 80.8 μ L) and Cholesterol (3.57 mM, 69.2 μ L), including Texas Red-DHPE (0.8 mol %), were used. Please see for the details of the section 3.2 for the details of the preparation process. Briefly, the process is described below. Together with a pre-cleaned ITO coated slide, the lipid-coated ITO slide was arranged to form a capacitor cell. The chamber was filled with 200 mM sucrose solution. A sinusoidal potential with 1.0 V amplitude (peak-to-peak) is applied with a frequency of 10 Hz for 2 hours and of 2 Hz for 1 hour. During the hydration, the temperature of DOPC and DPPC/Cholesterol sample solution was kept at room temperature and 55°C, respectively.

GM1 addition

DOPC or DPPC/Cholesterol (7:6) solution was mixed with GM1 solution of varying concentration; 50 μ M to 250 μ M. After incubating for two hours, the recovered GUV solution was mixed with slightly hypertonic glucose solution (from 210 mM to 230 mM) upon observation, which gives GUVs the small excess area and sink them to the bottom of a observation chamber. The mixture was put on-to a glass base dish with 35 mm diameter (Iwaki) and sealed with the slide glass. After equilibrium, the experiments were performed at room temperature.

Florescent microscopic observation

Please see the section 3.2.

Flickering analysis

Typically, in case of phase-contrast imaging, the contour of fluctuating GUVs is reconstructed by finding the inflection point in the radial intensity profile, as in reference [88]. In case of fluorescence imaging, the maximum of the intensity profile is commonly used to mark the membrane position. Here, we designed a Matlab algorithm to reconstruct the contour of GUVs from their equatorial cross section with sub-pixel precision, independently on the imaging mode employed. The algorithm proceeds following these steps:

1. The position of the centre, and a rough-guess value for the radius R of the GUV are automatically detected on the first frame of the video by processing of the thresholded image (see Fig. 5.1a).
2. The image of the radial profile $C(r, \varphi)$, where r is the distance from the centre and φ the azimuthal angle, is selected within an annular region of user-defined width that contains the membrane. The annular region is then mapped onto a rectangular stripe using a cubic interpolation (see Fig. 5.1b, top).

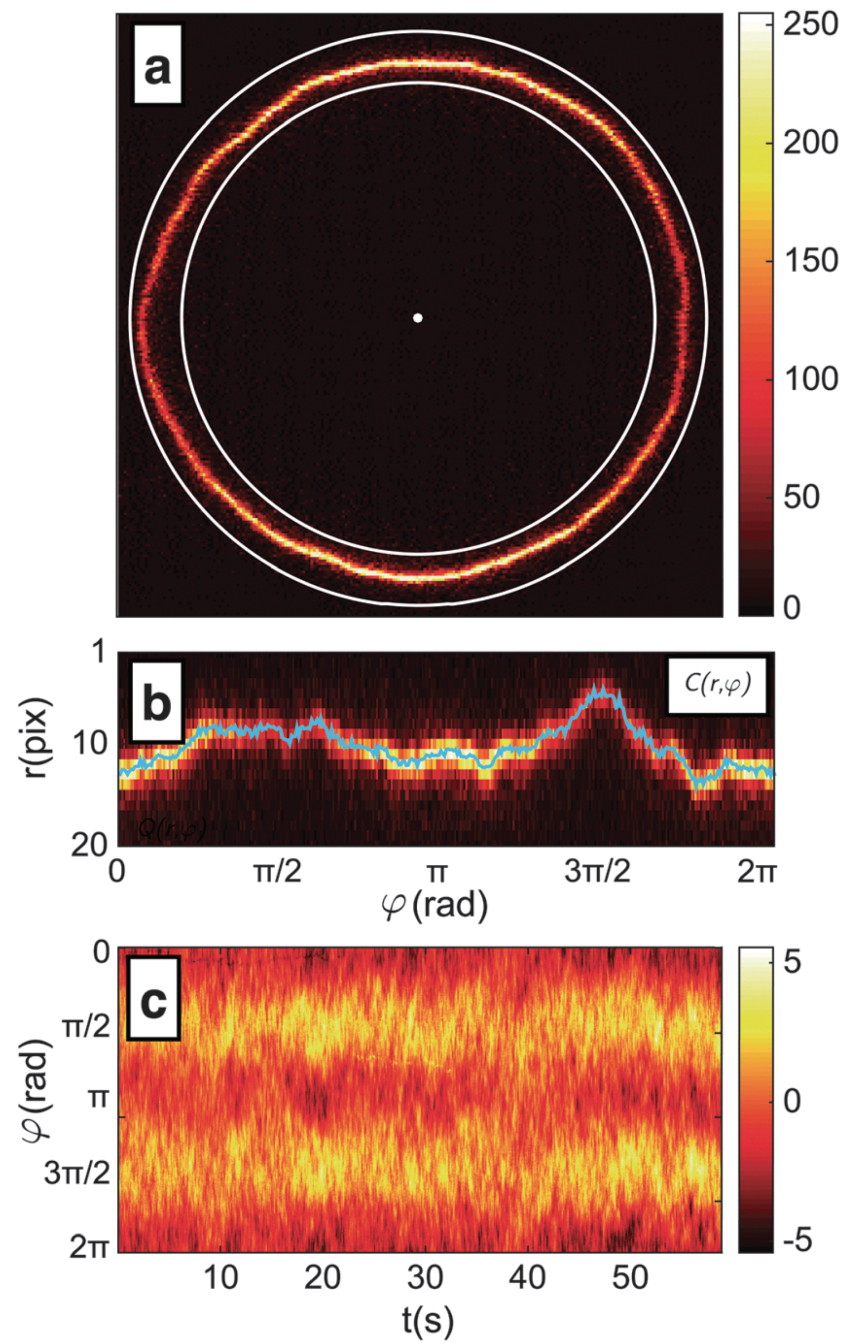


FIGURE 5.1: Intermediate steps of the contour detection algorithm. (a) The position of the centre and the mean radius R of the vesicle define an annular region in the frame; (b) the annular region $C(r, \varphi)$ extrapolated from the frame and fitted to find the membrane position with sub-pixel precision, as described in the text. The resulting contour is reported as a blue line; (c) colormap representation of the membrane fluctuations relative to the average radius. The colorbar reports the relative fluctuation as percentage in the range -5% , $+5\%$

3. For each φ , the position of the membrane is roughly located as the maximum

of $C(r, \varphi)$. The precision of this estimate is limited by the pixel size and highly susceptible to noise.

4. For each φ , the contour location is refined by evaluating the centroid of $C(r, \varphi)$ within an interval of 6 pixel centred around the maximum found in step 3. This interval is chosen as about 3 times the spatial resolution of the microscope to include the entire radial section of the membrane. The centroid calculation is iterated 5 times, each time by re-centring the 6-pixel interval around the centroid found in the previous step. This procedure allows for sub-pixel resolution.
5. The algorithm cycles three times from point 2 to 4, each time refining the estimate of the vesicle centre and mean radius using the obtained contour. The resulting radial profile $r(\varphi)$ is shown in Fig. 5.1b.

The procedure is repeated for every frame in a video of the fluctuating membrane, each time using the center and radius values of the previous frame as the starting point of the algorithm. An example of the temporal evolution of the membrane radial profile $r(\varphi)$ is shown in Fig. 5.1c. The spectrum of the thermal fluctuations of the contour is calculated using Matlab Fast Fourier Transform algorithm, using the formula

$$\langle |h^2(q_x)| \rangle = \frac{\langle |FFT(R(\varphi, t))|^2 \rangle_t}{M^2}$$

where M is the number points used to map the contour, and then averaged over the ensemble of frames.

5.3 Experimental results

The bending moduli in DOPC and DPPC/Cholesterol (7/6) GUVs were measured by flickering analysis of the equatorial cross sections of GUVs, respectively. The bending moduli were extracted by fitting the power spectra of the thermal fluctuations, with the following function derived in the section 2,

$$\langle h(q_x)^2 \rangle = \frac{1}{L} \frac{k_B T}{2\sigma} \left[\frac{1}{q_x} - \frac{1}{\sqrt{q_x^2 + \frac{\sigma}{\kappa}}} \right], \quad (5.1)$$

where L is the contour length of the equatorial cross-section of the GUV, σ is the membrane tension, κ is the bending modulus, and q_x is the wave vector evaluated along the contour. Of the discrete set of wave vectors $q_x(n)$, modes with $n < 6$ are excluded from the analysis. Modes with $n = 0$ and $n = 1$ correspond to size changes and translation of the GUV. Modes with $n > 2$ describe thermal fluctuations. However, Eq (5.1) is derived for a planar membrane, and should not be used to describe modes with $n < 6$, which are influenced by the spherical geometry of the GUV [88]. For our analysis we fit the spectra for modes $6 \leq n \leq 13$.

In Fig. 5.2(a) blue circles mark the bending modulus of DOPC GUVs with error bars, measured as a function of added GM1 concentration. The numbers of the investigated DOPC GUVs are 9 for 0 μ M, 11 for 50 μ M, 10 for 100 μ M, and 10 for 250

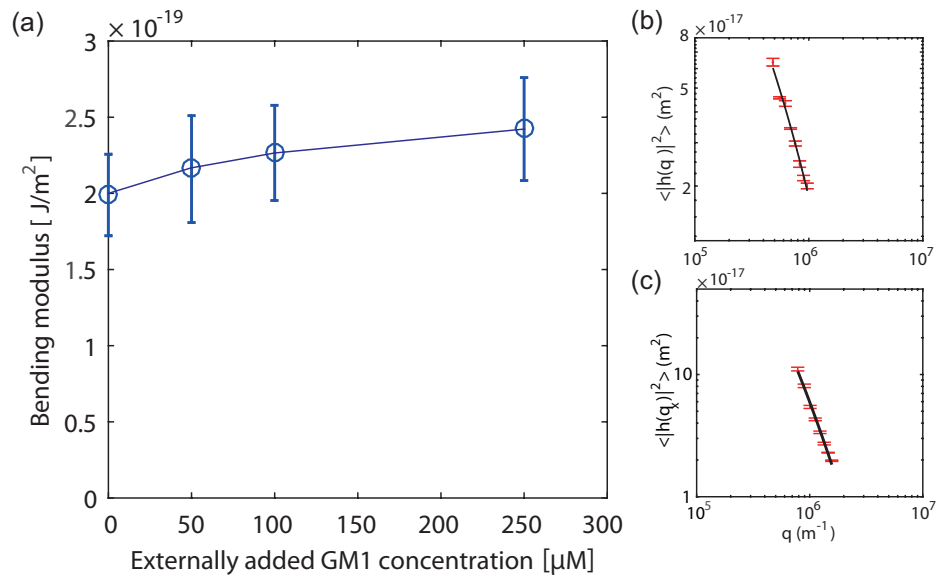


FIGURE 5.2: Dependence of the bending moduli of DOPC GUVs on GM1 concentration. (a) The bending moduli measured by flickering analysis with error bars are shown as a function of GM1 concentration. The numbers of the investigated DOPC GUVs are 9 for 0 μM , 11 for 50 μM , 10 for 100 μM , and 10 for 250 μM , respectively. (b) Fluctuation-amplitude spectra with error bars for a GUV, to which no GM1 was added, is shown. (c) Fluctuation-amplitude spectra with error bars for a GUV, to which 250 μM GM1 was added, is shown. Black-solid lines indicate fits by Eq (5.1).

μM , respectively. In Fig. 5.2(b) and (c), we show examples of power spectra with error bars for different externally added GM1 concentration ($0 \mu\text{M}$ in (b) and $250 \mu\text{M}$ in (c)), fitted by Eq (5.1), respectively. In Fig. 5.3(a) red circles mark the bending modulus of DPPC/Cholesterol (7/6) GUVs with error bars, measured as a function of added GM1 concentration. The numbers of the investigated DPPC/Cholesterol (7/6) GUVs are 11 for $0 \mu\text{M}$, 9 for $50 \mu\text{M}$, 9 for $100 \mu\text{M}$, and 11 for $250 \mu\text{M}$, respectively. In Fig. 5.3(b) and (c), we show examples of power spectra with error bars for different externally added GM1 concentration ($0 \mu\text{M}$ in (b) and $250 \mu\text{M}$ in (c)), fitted by Eq (5.1), respectively. In Fig. 5.4 we show the ratio of the bending modulus of DPPC/Cholesterol (7/6) to that of DOPC GUVs as a function of added GM1 concentration.

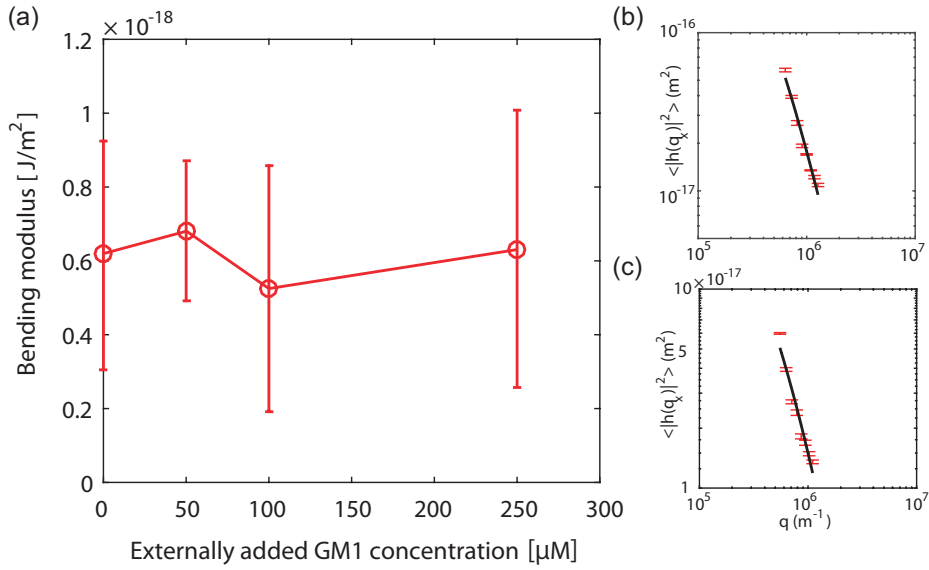


FIGURE 5.3: Dependence of the bending modulus of DPPC/Cholesterol (7/6) GUVs on GM1 concentration. (a) The bending moduli measured by flickering analysis with error bars are shown as a function of GM1 concentration. The numbers of the investigated DPPC/Cholesterol GUVs are 11 for $0 \mu\text{M}$, 9 for $50 \mu\text{M}$, 9 for $100 \mu\text{M}$, and 11 for $250 \mu\text{M}$, respectively. (b) Fluctuation-amplitude spectra with error bars for a GUV, to which no GM1 was added, is shown. (c) Fluctuation-amplitude spectra with error bars for a GUV, to which $250 \mu\text{M}$ GM1 was added, is shown. Black-solid lines indicate fits by Eq (5.1).

5.4 Discussions

The measured bending moduli of DOPC and DPPC/Cholesterol GUVs, to which no GM1 was added, are quite close to those measured in the similar experimental conditions using a different method [89]. As shown in Fig. 5.2(a), the bending moduli of DOPC GUVs consistently display a weak dependence on externally added GM1 concentration in the tested range, which might be attributed to the stronger

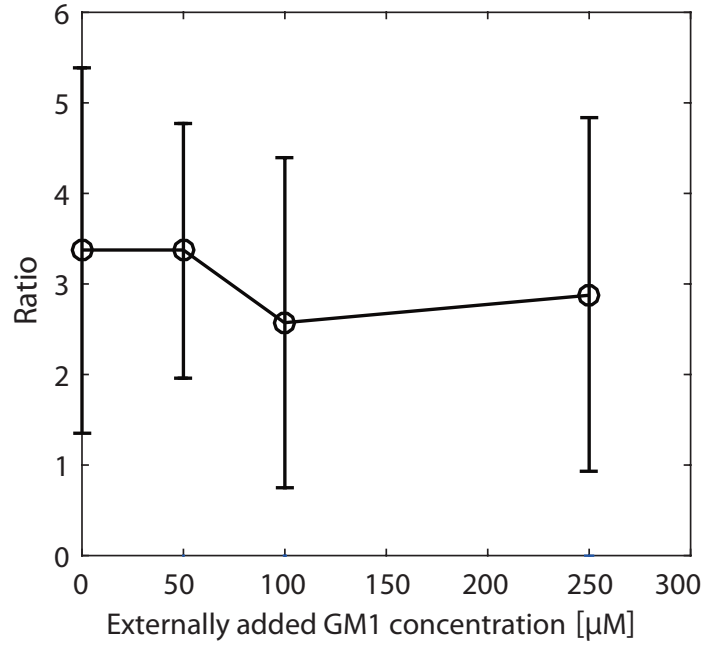


FIGURE 5.4: The ratio of the bending modulus of DPPC/Cholesterol (7/6) to that of DOPC GUVs as a function of added GM1 concentration.

interaction between saturated hydrocarbon chains of GM1. On the other hand, as shown in Fig. 5.3(a), the bending moduli of DPPC/Cholesterol GUVs represent no dependence on the added GM1 concentration in the tested range. One notes that the magnitude of each error bar is larger than that in DOPC GUVs, which would be ascribed to the polydispersity of the ratio between the concentration of DPPC and Cholesterol in each electroformed GUV. As shown in Fig. 5.4, the ratio of the bending modulus of DPPC/Cholesterol (7/6) to that of DOPC GUVs is apparently independent of externally added GM1 concentration. The average values of the ratio are approximately 3 and the error bars almost cover the measured values in the similar experimental conditions [18, 89].

5.5 Summary

The effects of GM1 inserted into DOPC and DPPC/Cholesterol (7/6) GUVs on the bending modulus were investigated by flickering measurements, respectively, suggesting that the ratio of the bending modulus of DPPC/Cholesterol (7/6) GUVs to that of DOPC GUVs is apparently independent of externally added GM1 concentration. Therefore, we can conclude that our assumption in the numerical analysis would be valid in the tested GM1 concentration range that the ratio of the bending moduli in the L_o and L_d phases is not affected by inserted GM1.

Chapter 6

Micro-domain size depending the degree of lipid asymmetry

6.1 Introduction

In Chapter 3, we showed the experimental results of the transitions from macro- to micro-phase separation via a metastable stripe-phase in multi-component GUVs. In Chapter 4, numerical analysis gave us insights into the mechanisms, suggesting that the local spontaneous curvature due to the local asymmetric lipid composition plays the essential role in the transitions. In Chapter 5, an essential assumption in our numerical analysis was confirmed experimentally. According to the results, we expect that the micro domains should become smaller with the increasing asymmetry of the lipid composition. However, there is no direct evidence. If the expectation is proved in the experiments, the quality of the underlying assumptions in the numerical simulations is confirmed. In the chapter, we investigate the expectation experimentally and numerically.

6.2 Materials and methods

Materials

Please see the section 3.2.

GUV preparation

GUVs were prepared by electroformation from a mixture of 35 mol % DOPC, 35 mol % DPPC, and 30 mol % Cholesterol. Please see the section 3.2 for the details of the preparation process. Briefly, the process is described below. Together with a pre-cleaned ITO coated slide, the lipid-coated ITO slide was arranged to form a capacitor cell. The chamber was filled with 400 mM sucrose solution. A sinusoidal potential with 1.0 V amplitude (peak-to-peak) is applied with a frequency of 10 Hz for 2 hours and of 2 Hz for 1 hour. During the hydration for three hours, the temperature of the sample solution was kept at 55°C.

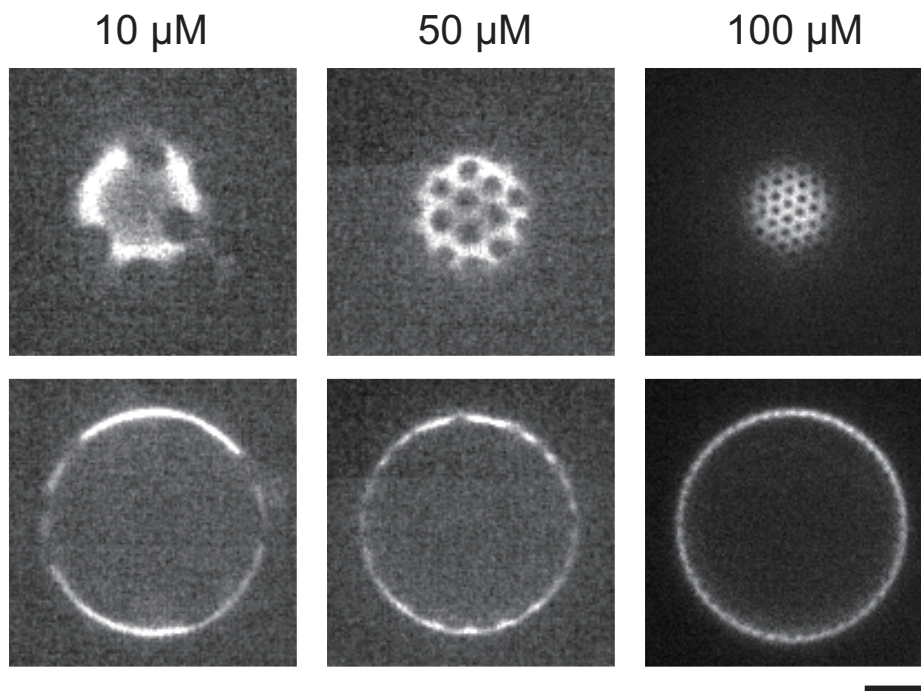


FIGURE 6.1: Confocal microscope images of top (upper) and cross-sectional (lower) views of GUVs with three different added GM1 concentrations (10 μM , 50 μM , and 100 μM). White regions represent L_d phase. The scale bar is 5 μm .

GM1 addition

The solution of the fully phase-separated GUVs was mixed with GM1 solution of varying concentration; 10 μM to 250 μM . After mixing, the GM1 solution was left for at least six hours. During the time, the phase separation morphologies of some GUVs transits from macro-phase to micro-phase. The GUV solution was mixed with isotonic glucose solution upon observation, which sink them to the bottom of an observation chamber due to the density difference between sucrose and glucose. The mixture was put on-to a glass base dish with 35 mm diameter (Iwaki) and sealed with the slide glass.

Florescent microscopic observation

Please see the section 3.2.

6.3 Experimental results

In FIG. 6.1, we show examples of the confocal microscope images of the top and cross-sectional views of the GUVs with three different added GM1 concentrations (10 μM , 50 μM and 100 μM). The ratio of the average size of micro domains $2r$ to the vesicle radius R was measured as a function of the mole fraction of GM1, using GUVs with a radius of $7.5 \pm 2.5 \mu\text{m}$ to avoid the dependence of the vesicle size

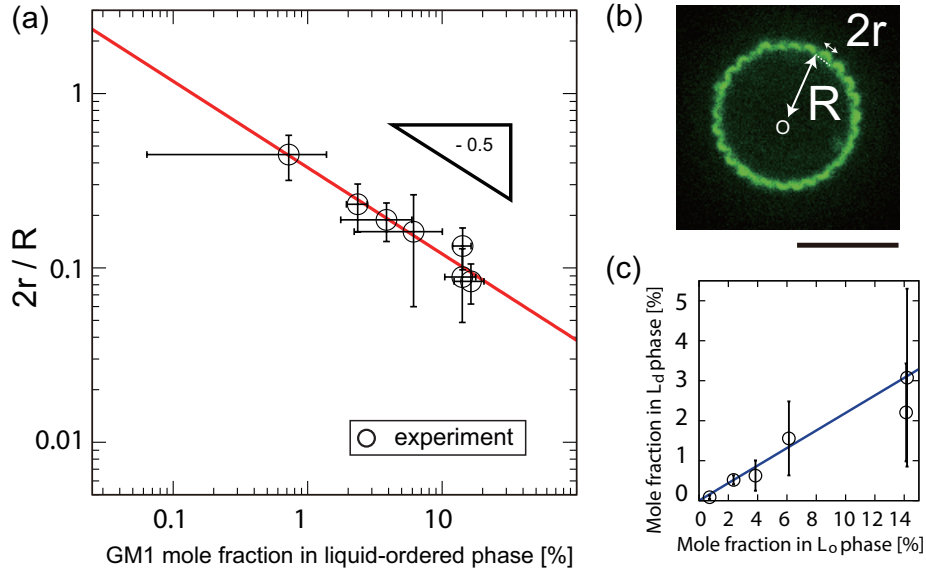


FIGURE 6.2: Power law behavior. (a) Nontrivial power law behavior between $2r/R$ and the GM1 mole fraction in L_o phase. The experimental results (open circles) are fitted by the red-solid power law line, whose exponent is -0.5 ± 0.03 . (b) Definitions of the domain size $2r$ and the L_d matrix radius R in the experiment. The scale bar is $10 \mu\text{m}$. (c) The GM1 mole fraction in the L_o and L_d phases are linearly related with a best-fit slope of 0.22 ± 0.02 (blue-solid line).

on the ratio (FIG. 6.2). The mole fractions of GM1 in the L_d and L_o phases were estimated by comparing the mean intensities of GM1 prepared in the film and introduced from outside (see supplementary information in Chapter 3: “Quantitative estimation of the mole fractions of inserted GM1”). The GM1 concentration ratio in the L_d phase to the L_o phase was then estimated as 0.22 ± 0.02 , as shown in FIG. 6.2(c). As expected, the ratio $2r/R$ decreases as the mole fraction of GM1 increases. The nontrivial power law with the exponent ($= -0.5 \pm 0.03$) between $2r/R$ and the mole fraction of GM1 in the L_o phase is intriguingly observed (FIG. 6.2(a), red-solid line), where the data were fitted by a power law line.

6.4 Numerical results

In numerical analysis, we also investigated whether the higher degree of lipid asymmetry makes smaller micro domains. For investigating this, we solved the time-dependent Ginzburg-Landau model, which describes intramembrane phase separation and the bending elastic membrane as well as Chapter 4. Please see Chapter 4 for the details of the theoretical analysis. In our simulation, the spontaneous curvature of the fully phase-separated vesicle before adding GM1 ($\tilde{t} < 0$) was set to zero, i.e., $\tilde{H}_{sp}^{(0)} = 0$ and $\tilde{H}_{sp}^{(1)} = 0$. The vesicle was sufficiently relaxed to an equilibrium state. At $\tilde{t} = 0$ we assumed that GM1 was instantly inserted into the

outer layer, changing the spontaneous curvature in each domain. Setting the non-zero spontaneous curvatures at $\tilde{t} = 0$, we measured the ratio of the average size of micro domains $2r$ to the vesicle radius R after enough time to transit from the macro- to micro-phase. The non-zero spontaneous curvatures in each domain were estimated using (i) the experimentally observed linear relation between the mole fraction of GM1 in the L_o and L_d phases (FIG. 6.2(c)) and (ii) the two assumptions that the spontaneous curvature of a monolayer is the linear function of the mole fractions of the components [84, 85] and the spontaneous curvature of a bilayer is computed as $H_{sp} = (H_{sp}^{out} - H_{sp}^{in})/2$, where H_{sp}^{in} and H_{sp}^{out} are the spontaneous curvatures of the inner and outer monolayers, respectively. We show examples of the GUVs with $(\tilde{H}_{sp}^{(0)}, \tilde{H}_{sp}^{(1)}) = (0.095, 0.045)$ and at $\tilde{t}=500$, with $(\tilde{H}_{sp}^{(0)}, \tilde{H}_{sp}^{(1)}) = (0.114, 0.054)$ and at $\tilde{t} = 300$, and with $(\tilde{H}_{sp}^{(0)}, \tilde{H}_{sp}^{(1)}) = (0.171, 0.081)$ and at $\tilde{t} = 50$ in FIG. 6.3. Moreover, their profiles of ϕ are described as a function of the angle θ . The GM1 mole fraction in the L_o phase corresponds to 5 mol %, 6 mol %, and 9 mol %, respectively. We performed not 3D simulations but 2D simulations because the computation cost is reduced in the 2D simulations and the analysis is easier to calculate the parameter $2r/R$. Surprisingly, the power law behavior with the exponent $(= -1.99 \pm 0.09)$ is also confirmed by the numerical simulations (FIG. 6.4, blue-dashed line), where the data were fitted by a power law line. The parameters were set to $N = 1000$, $\tilde{\kappa}_0 = 300$, $\tilde{\kappa}_1 = 1/9$, $\tilde{\Gamma} = 1$ and $(\tilde{H}_{sp}^{(0)}, \tilde{H}_{sp}^{(1)}) = (0.095, 0.045)$, $(0.114, 0.054)$, $(0.133, 0.063)$, $(0.171, 0.081)$, $(0.209, 0.099)$, $(0.247, 0.117)$, $(0.286, 0.134)$ and $(0.324, 0.152)$.

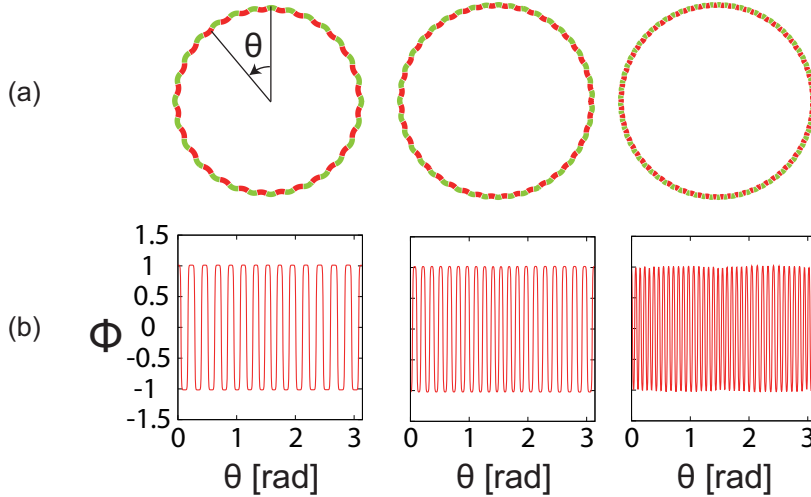


FIGURE 6.3: From left to right: (a) Vesicles and (b) ϕ -profiles with $(\tilde{H}_{sp}^{(0)}, \tilde{H}_{sp}^{(1)}) = (0.095, 0.045)$ and at $\tilde{t}=500$ (left), with $(\tilde{H}_{sp}^{(0)}, \tilde{H}_{sp}^{(1)}) = (0.114, 0.054)$ and at $\tilde{t} = 300$ (middle), and with $(\tilde{H}_{sp}^{(0)}, \tilde{H}_{sp}^{(1)}) = (0.171, 0.081)$ and at $\tilde{t} = 50$ (right), respectively. The domains where $\phi \geq 0$ (rich in DPPC) and $\phi < 0$ (rich in DOPC) are shown in green and red, respectively. The other parameters were set to $N = 1000$, $\tilde{\kappa}_0 = 300$, $\tilde{\kappa}_1 = 1/9$, and $\tilde{\Gamma} = 1$, respectively

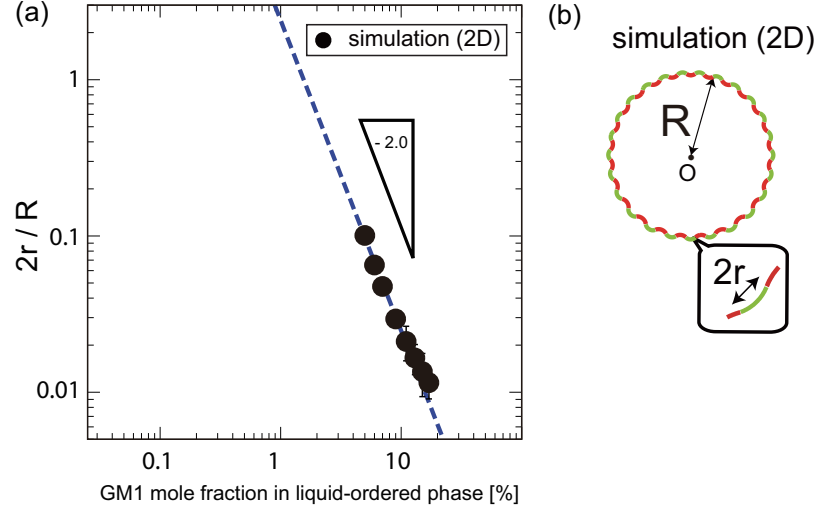


FIGURE 6.4: Power law behavior. (a) Nontrivial power law behavior between $2r/R$ and the GM1 mole fraction in L_o phase. The numerical results (closed circles) are fitted by the blue-dashed power law line, whose exponent is -1.99 ± 0.09 . The parameters were set to $N = 1000$, $\tilde{\kappa}_0 = 300$, $\tilde{\kappa}_1 = 1/9$, $\tilde{\Gamma} = 1$ and $(\tilde{H}_{sp}^{(0)}, \tilde{H}_{sp}^{(1)}) = (0.095, 0.045), (0.114, 0.054), (0.133, 0.063), (0.171, 0.081), (0.209, 0.099), (0.247, 0.117), (0.286, 0.134)$ and $(0.324, 0.152)$. (b) Definitions of the domain size $2r$ and L_d matrix radius R in the simulation.

6.5 Discussions

We demonstrated that the micro domains become smaller with the increasing asymmetry of the lipid composition in both of the experiments and 2D numerical simulations, confirming the quality of the underlying assumptions in the numerical analysis. Thus, we could say that the degree of lipid asymmetry is a key parameter in determining the domain size. Moreover, we discovered nontrivial power laws between the degree of lipid asymmetry and the micro domain size in both of the experiments and 2D numerical simulations, respectively. However, the origin of the nontrivial power law behaviors and the discrepancy between the exponents still remain poorly understood.

6.6 Summary

As expected by our findings in Chapters 3, 4, and 5, we revealed that the higher degree of lipid asymmetry produces smaller micro domains in both of the experiments and numerical simulations, confirming the quality of the underlying assumptions. Moreover, we discovered a nontrivial power law between the degree of *lipid asymmetry* and the micro domain size.

Part III

Direct measurement of DNA-mediated adhesion between lipid bilayers

Chapter 7

Direct measurement of DNA-mediated adhesion between lipid bilayers

7.1 Introduction

For the last several decades, the physics of single liposome to mimic the fundamental structure of cell membranes has been revealed experimentally, theoretically, and numerically, and thus there exists rich knowledge with regard to it. In contrast, the physics of the complex system has been still poorly understood, which is composed of the adhesive-functionalized liposomes to mimic adhered cells to other cells and/or extracellular matrix. In nature, cells interact with other materials through glycolipids [31] and/or proteins, including selectins, integrins, and cadherins [32–34]. However, it is almost impossible to tune the interaction between adhesive macromolecules extracted from cells, preventing from understanding the physics of the complex system.

Since nearly two decades ago, artificial adhesion techniques by programmable multivalent interactions between sticky DNAs have been developed. Introduced by the seminal works of Mirkin [90] and Alivisatos [91], who demonstrated the selective DNA-mediated self-assembly of gold nanoparticles, this approach has been optimised to the point of mastering the structure of multicomponent crystal lattices [92–96] and amorphous materials [97–99]. Applications of these self-assembly strategies span from photonics and plasmonics [100], to biosensing [101], and gene therapy [102–104]. An abundance of experimental studies called for the development of models to understand the complex statistical mechanics of DNA-mediated multivalent interactions between solid particles [105–111]. More recently, the same self-assembly strategy has been applied to compliant Brownian units, including emulsion droplets [112, 113], and lipid vesicles [114–118]. The advantages of the adhesion techniques by synthesized DNA over natural glues extracted from cells are the selectivity and tunability of the adhesive interaction due to chemosynthesis, enabling to investigate the complex phenomenology quantitatively.

P. Cicutta *et al.*, who are our collaborators, recently demonstrated that the coupling between mechanical deformability of Giant-Unilamellar-Vesicles (GUVs) and DNA-mediated interactions, results in unexpected emergent response to temperature changes, leading to negative thermal expansion and tuneable porosity

of DNA-GUV assemblies [37]. By means of an analytical theory, that jointly describes the geometrical features of the GUVs and the statistical mechanics of DNA tethers [37, 119], they rationalised experimental evidence and highlighted the importance of translational entropy in coupling the DNA-binding free energy to the morphology of the substrates. Nonetheless, a further experimental investigation is necessary to assess all the aspects of the complex phenomenology of DNA-GUV conjugates, and test the accuracy of the theoretical framework applied to their description. In particular, the quantitative characterisation of the melting transition would represent new strong tests of the current understanding.

In this chapter, we present experiments aimed at measuring the temperature-dependence of the membrane tension induced on giant liposomes by DNA-mediated adhesion. Membrane tension is accessed by *flickering* measurements, in which we reconstruct the spectrum of thermal fluctuations of the liposomes at the equatorial cross-sections [42, 88, 120]. This is a classic technique widely used to study vesicle tension and bending rigidity, as introduced in Chapter 2.1. To make these measurements possible, we adopt an experimental geometry in which liposomes do not interact with each other, instead they adhere to Supported Lipid Bilayers (SBLs) fabricated on rigid glass substrates, as demonstrated in FIG. 7.1. This allows imaging of the equator of the vesicle through confocal microscopy. Various morphological observables are also precisely assessed via 3-dimensional confocal imaging. Finally, the use of suitably labelled DNA tethers enables the direct assessment of the relative number of formed DNA bonds by means of in-situ Förster Resonant Energy Transfer (FRET) spectroscopy. Experimental results are compared to predictions from the analytical theory mainly developed by our collaborator, B. M. Mognetti, extended from ref. [37] to apply to this new geometry.

The remainder of this article is structured as follows. First we describe the experimental setup, including sample preparations protocols, materials, imaging and image analysis techniques. Then, we briefly outline the theoretical model focusing on the novel aspects introduced here for the GUV-plane geometry. Finally, we discuss the experimental results and compare them with theoretical predictions.

7.2 Experimental

FIGURE 7.1 shows a schematic of a DNA-functionalised GUV adhering to a DNA-functionalised SBL. GUVs and SBLs are prepared and separately functionalised with cholesterol labeled DNA constructs. The hydrophobic cholesterol inserts into the lipid bilayer allowing DNA tethers to freely diffuse. Connected to the cholesterol anchor there is a section of length $L = 9.8$ nm (29 base pairs [121]) of double-strands DNA (dsDNA), terminating in a single-stranded DNA (ssDNA) *sticky end*, which mediates the attractive interactions. To further facilitate the pivoting motion of the DNA tethers, 4 unpaired adenine bases are left between the cholesterol anchor and the dsDNA spacer. One unpaired adenine is left between the dsDNA spacer and the sticky end. Two mutually complementary sticky ends are used: a and a' , of 7 bases each (see FIG. 7.1). GUVs and SBLs are functionalised with equal molar fractions of both a and a' . Tethers can therefore form intra-membrane

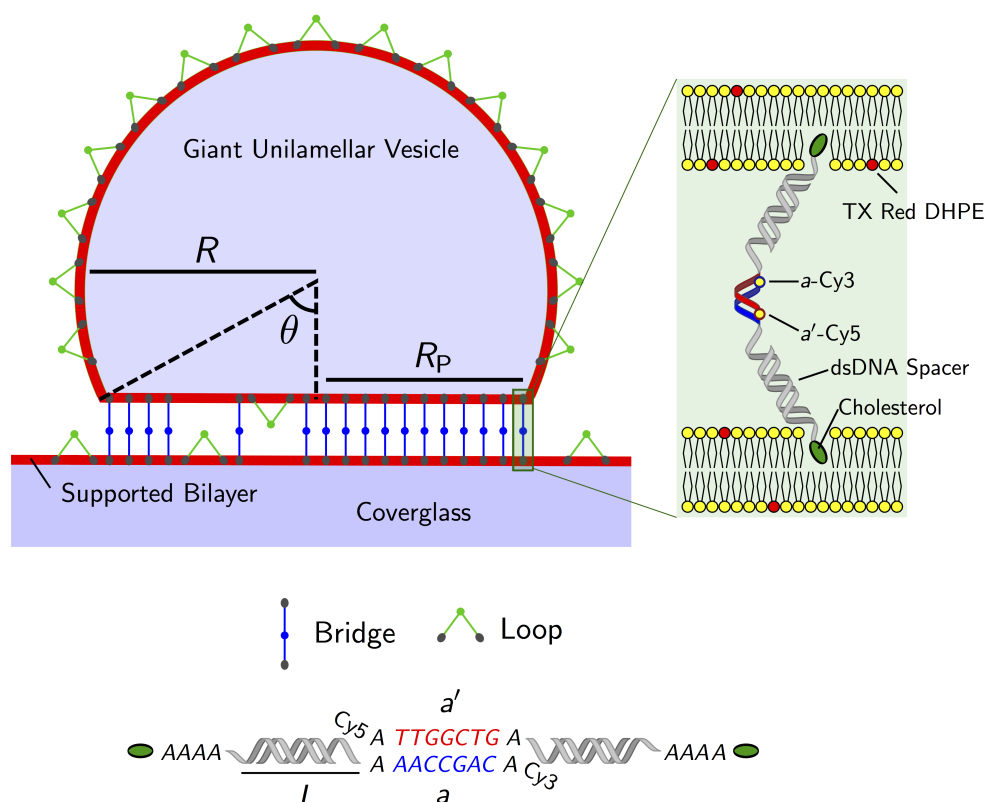


FIGURE 7.1: Not-to-scale schematics of a DNA-functionalised GUV adhering to a supported bilayer and details of the functionalisation.

loops and inter-membrane *bridges*. The latter are responsible for the observed adhesion and are confined within the contact area between the GUVs and the SBLs. We use FRET spectroscopy to estimate the fraction of formed DNA bonds. To enable these measurements the termini of the sticky ends a and a' are functionalised with a Cy3 (indocarbocyanine) and a Cy5 (indodicarbocyanine) molecules respectively, spaced by an unpaired adenine base. Note that the sticky ends used here are shorter than those adopted in our previous study [37] (7 base-pairs instead of 9), this choice was made to lower the melting temperature of the DNA to within an experimentally accessible temperature, and reduce the overall strength of the DNA-mediated adhesion, making it easier to measure by flickering spectroscopy.

Materials and sample preparation

GUVs electroformation

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC, Avanti Polar Lipids) GUVs are prepared by standard electroformation in 300 mM sucrose (Sigma Aldrich) solution in double-distilled water [122, 123]. Full details can be found in ref. [37].

Supported bilayers

DOPC supported bilayers (SBLs) are formed by rupture of Small Unilamellar Vesicles (SUVs ~ 100 nm) on the hydrophilised glass bottom of sample chambers [124, 125]. SUVs are prepared by standard extrusion. Briefly, 200 μ l of 25 mg/ml DOPC solution in chloroform are left to dry in a glass vial, then hydrated by adding 500 μ l of 300 mM sucrose solution and mixed by vortexing for at least 5 minutes. The solutions are then transferred in plastic vials and treated with 5 cycles of rapid freezing/unfreezing by alternatively immersing the vial in baths of liquid nitrogen and warm water [116]. Extrusion is carried out using a hand-driven mini-extruder (Avanti Polar Lipids) with a polycarbonate track-etched membrane (100 nm pores, Whatman). To facilitate rupture of the SUVs and bilayer formation, the extruded solution is diluted in a 1:9 ratio in iso-osmolar solution containing TE buffer (10 mM tris(hydroxymethyl) aminomethane, 1 mM ethylenediaminetetra acetic acid, Sigma Aldrich), 5 mM $MgCl_2$ and 272 mM glucose (Sigma Aldrich).

Sample chambers are obtained by applying adhesive silicone-rubber multi-well plates (6.5 mm $\times$ 6.5 mm $\times$ 3.2 mm, Flexwell, Grace Biolabs) on glass coverslips (26 $\times$ 60 mm, No.1, Menzel-Gläser), cleaned following a previously reported protocol [97]. To form SBLs, the glass bottom of the cells is hydrophilised by plasma cleaning on a plasmochemical reactor (Femto, Diener electronic, Germany), operated at frequency of 40 kHz, pressure of 30 Pa, and power input of 100 W for 5 minutes. Within 5 minutes from plasma cleaning, each cell is filled with 100 μ l of diluted SUV solution and incubated for at least 30 minutes at room temperature to allow bilayer formation. To remove excess lipid and magnesium, the cells are repeatedly rinsed with the experimental solution (TE buffer, 100 mM NaCl, 87 mM glucose). Care is taken to always keep the bilayers covered in buffer to avoid exposure to air.

For experiments not involving FRET spectroscopy SUVs and SBLs are stained with 0.8-1.2% molar fraction of Texas Red 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt (Texas Red DHPE, Life Technologies).

DNA preparation

The DNA tethers are pre-assembled from two ssDNA strands, one of them (i) functionalized with a cholesterol molecule and the second (ii) carrying the sticky end:

i 5'-CGT **GCG CTG GCG TCT GAA AGT CGA TTG CG** AAAAA [Cholesterol TEG] -3'

ii 5'-GC **GAA TCG ACT TTC AGA CGC CAG CGC ACG A** [Sticky End] A
Cy3/Cy5 -3'.

The bold letters indicate the segments forming the dsDNA spacer, the italic letters the inert flexible spacers. DNA is purchased lyophilized from Integrated DNA Technologies, reconstituted in TE buffer, aliquoted, and stored at -20 °C. For assembling the constructs, we dilute equal amounts of the two single strands, *i* and

ii, to 1.6 μM in TE buffer containing 100 mM NaCl. Hybridization is carried out by ramping down the temperature from 90°C to 20°C at a rate of $-0.2^\circ\text{C min}^{-1}$ on a PCR machine (Eppendorf Mastercycler) [37].

Membrane functionalisation.

Functionalisation of the supported bilayers is carried out by injecting 90 μL of iso-osmolar experimental solution (TE buffer, 87 mM glucose, 100 mM NaCl) containing overall X moles of DNA constructs into each of the silicone-rubber cells, with equal molarity of a and a' strands. Similarly, GUVs are functionalised by diluting 10 μL of electroformed vesicle solution in 90 μL of iso-osmolar experimental solution containing X moles of DNA constructs. GUVs and SLBs are incubated for at least 1 hour at room temperature to allow grafting. After incubation, 10 μL of the liposome solution are injected into the sample chambers, which are immediately closed with a second clean coverslip and sealed using rapid epoxy glue (Araldite). Care is taken to prevent the formation of air bubbles. Sedimentation of the GUVs results in the formation of an adhesion patch between GUVs and supported bilayer. In a typical sample a fraction of the GUVs is found to form clusters. We limit our analysis to isolated GUVs.

We tested samples at different DNA concentrations, obtained by setting X to $X_{\text{low}} = 0.09$, $X_{\text{med}} = 0.9$ and $X_{\text{high}} = 2.7$ pmoles. We have previously quantified the surface coverage of GUVs functionalised with $X_{\text{med}} = 0.9$ pmoles in $\rho_{\text{DNA}}^{\text{med}} = 390 \pm 90 \mu\text{m}^{-2}$ [37]. Here we proportionally assume $\rho_{\text{DNA}}^{\text{low}} = 39 \pm 9 \mu\text{m}^{-2}$, $\rho_{\text{DNA}}^{\text{high}} = 1200 \pm 300 \mu\text{m}^{-2}$. Having estimated that the overall surface of the SBL is approximately equal to the surface of the GUVs used for each sample, we assume equal DNA coverages for the SBL. This assumption is confirmed by fluorescence emission measurements: at sufficiently high temperature, when no DNA bridges are formed between GUVs and SBL, DNA is uniformly distributed on both interfaces. In this regime the fluorescence emission from DNA located within the contact area between a GUV and the SBL approximately equals twice the intensity measured on the free SBL (2.1 ± 0.1), confirming that GUVs and SBL have, within experimental errors, the same DNA coverage.

Imaging and image analysis

Imaging and temperature cycling.

The samples are imaged on a Leica TCS SP5 laser-scanning confocal microscope. Texas Red DHPE is excited with a He-Ne laser (594 nm). For FRET spectroscopy measurements, Cy3 is excited with an Ar-ion laser line (514 nm). The temperature of the sample is regulated with a home-made Peltier device controlled by a PID (proportional-integral-derivative) controller, featuring a copper plate to which the sample chamber is kept in thermal contact. Two thermocouples are used as temperature sensors. The first sensor, kept in contact with the copper plate, serves as a feedback probe for the PID controller. The second thermocouple is inserted in a dummy experimental chamber, filled with water, and used to precisely probe the

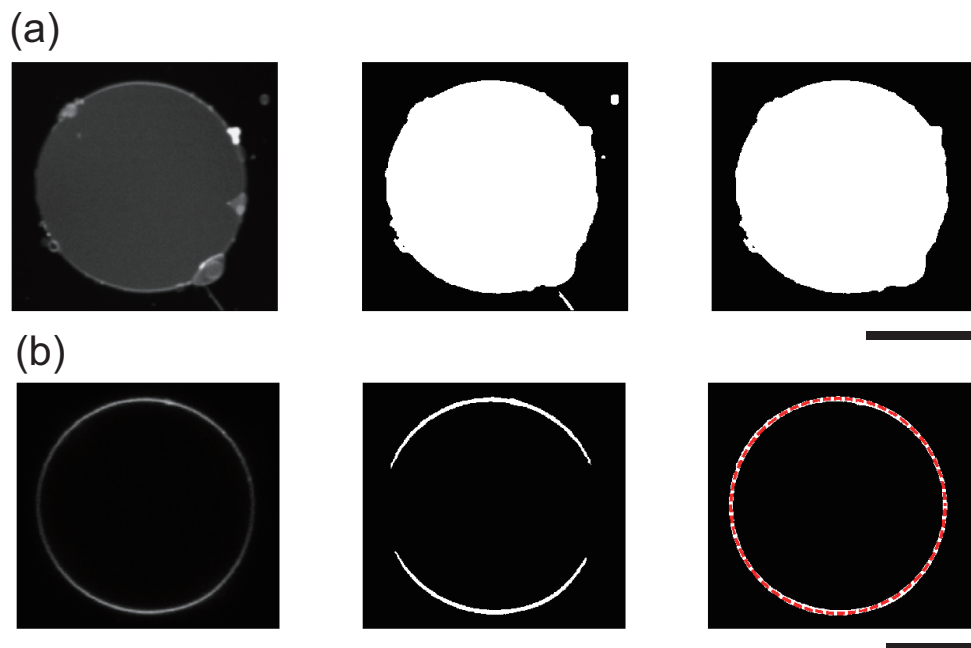


FIGURE 7.2: Image analysis for estimating the path-radius (a) R_p and (b) the vesicle radius R . (a) From left to right: the image identified as the plane of the SBL/adhesion path (left), its binary image (middle), and the binary image after removing some stuff (right). (b) From left to right: the image identified as the equatorial plane (left), its binary image (middle), and the binary image fitted with a red-dashed perfect circle (right). Scale bars are $10\ \mu\text{m}$.

temperature of the sample. For all the temperature-dependent experiments imaging is carried out using a Leica HCX PL APO CS 40×0.85 NA dry objective, to prevent heat dissipation.

The temperature-dependent morphology of adhering GUVs is captured via confocal z-stacks and reconstructed using a custom script written in Matlab. Briefly: Each z-stack contains a single adhering GUV. The plane of the SBL/adhesion patch is identified as the one with maximum average intensity, then the adhesion patch is reconstructed by thresholding, following the application of a bandpass filter to flatten the background and remove pixel-level noise and some stuff, as shown in FIG. 7.2(a). From the area A_p of the adhesion patch we extract the patch-radius as $R_p = \sqrt{A_p/\pi}$. The portion of the z-stack above the SBL is then scanned, and in each plane the contour of the vesicle, suitably highlighted by filtering and thresholding, is fitted with a circle, as shown in FIG. 7.2(b). The slice featuring the largest circle is identified as the equatorial plane, determining the vesicle radius R . The contact angle is derived as $\theta = \sin^{-1}(R_p/R)$ (see FIG. 7.1).

For flickering experiments, movies are recorded across the equatorial plane. Details of the flickering analysis are reported in the section 5.2. To improve the quality of the signal, imaging for morphological characterisation and flickering analysis is carried out on samples stained with Texas Red DHPE.

FRET spectroscopy measurements are carried out by performing a spectral

emission scan (λ -scan) of the contact area between an adhering GUV and the surrounding free SBL. While exciting the donor (Cy3), the emission of donor and acceptor is reconstructed by scanning the acquisition window from 530 to 785 nm, with a 6.75 nm binning. Similarly to the case of z-stacks, the adhesion patch is identified in each image by filtering and thresholding. For FRET imaging, non-fluorescent lipids are used in order to prevent undesired energy transfer between the bilayer and the DNA tethers.

Control experiments

Control experiments are performed to measure temperature-dependent membrane tension of non-adhering GUVs. Plain, non-functionalised, GUVs are imaged while laying on a glass substrate passivated with bovine serum albumin (BSA, Sigma Aldrich). Imaging is either carried out in confocal microscopy, as described above, or in epifluorescence microscopy using a Nikon Eclipse Ti-E inverted microscope, a Nikon PLAN APO 40 $\times$ 0.95 N.A. dry objective and a IICD Point Grey Reserach Grasshopper-3 camera. For control experiments GUVs are diluted in a 1:9 ratio in iso-osmolar glucose solution to enable sedimentation.

Flickering analysis

Please see Flickering analysis in the section 5.2.

7.3 Theoretical model

We briefly introduce a quantitative model describing the DNA-mediated adhesion of GUVs on flat supported bilayers. The model is adapted from reference [37], where we treated the case of two identical adhering GUVs. Let us consider the interaction between an infinite SBL and a GUV adhering to it

$$U = U_{\text{membrane}} + U_{\text{DNA}} + U_0, \quad (7.1)$$

where U_{membrane} accounts for the mechanical deformation of the GUV, U_{DNA} encodes for DNA-mediated adhesion, and U_0 is the reference energy, calculated for isolated GUV and SBL.

Membrane deformation

In Eq. (7.1), U_{membrane} summarizes three contributions: stretching energy, bending energy, and the entropic cost of suppressing thermal fluctuations of the contact area between the GUV and the SBL [126]. In the limit of *strong adhesion*, the stretching energy dominates over the other two contributions, which we can neglect [126]. As discussed later, for the case of DNA-mediated interactions, this condition is

generally verified at low enough temperature. We can rewrite

$$U_{\text{membrane}}(\theta) = U_{\text{stretching}}(\theta) = K_a \frac{[A(\theta) - \tilde{A}]^2}{2\tilde{A}}, \quad (7.2)$$

where K_a is the stretching modulus of the membrane, $A(\theta)$ is the overall (stretched) area of the GUV, and $\tilde{A}$ is the reference, un-stretched, area. In the limit of strong adhesion the GUV will take the shape of a truncated sphere with contact angle θ (FIG. 7.1), which we take as the independent variable of the model [126]. Within the assumption of constant inner volume $V = 4\pi R_0^3/3$, where R_0 is a reference radius, the total area A of the GUV and the adhesion patch area A_p can be expressed as a function of the contact angle θ (see supplementary information). The un-stretched vesicle area $\tilde{A}$ exhibits a strong temperature-dependence

$$\tilde{A} = A_0 [1 + \alpha(T - T_0)], \quad (7.3)$$

where α is the area thermal expansion coefficient [127] and T_0 is the *neutral temperature* of the GUVs, at which its reduced volume equals unity and $A = 4\pi R_0^2$ (see supplementary information).

DNA-mediated adhesion

We now focus on the DNA-mediated contribution to the interaction energy in Eq. (7.1): U_{DNA} .

Given that the persistence length of dsDNA is $\sim 50 \text{ nm} > L = 9.8 \text{ nm}$ [121], we can model the dsDNA spacers as rigid rods that, thanks to the fluidity of the membrane, can freely diffuse on the surface of the bilayers [37, 119]. Free pivoting motion is guaranteed by the flexibility of the joint between the cholesterol anchor and the dsDNA spacer (FIG. 7.1). As demonstrated in ref. [37], we can regard the sticky ends as point-like reactive sites, neglecting their physical dimensions. Moreover, we can safely assume that the distance between the adhering membranes within the contact area is equal to L [37]. Finally, we neglect excluded volume interactions between unbound DNA tethers [37, 128]. The last two assumptions guarantee a uniform distribution of unbound DNA tethers and loops over the GUV and SBL surfaces.

The free energy change associated to the formation of a single bridge (b) or a loop (ℓ) within the GUV is

$$\Delta G_{b/\ell} = \Delta G^0 - T\Delta S_{b/\ell}^{\text{conf}}, \quad (7.4)$$

where $\Delta G^0 = \Delta H^0 - T\Delta S^0$ is the hybridisation free energy of untethered sticky ends, which can be calculated from the nearest-neighbour thermodynamic model [129], eventually corrected to account for neighbouring non-hybridised bases [130]. In Eq. (7.4), the term $-T\Delta S_{b/\ell}^{\text{conf}}$ accounts for the confinement entropic loss taking place when tethered sticky ends hybridise [106, 108, 131], and can be

estimated as (see supplementary information)

$$\Delta G(\theta) = \Delta G^0 - k_B T \log \left[\frac{1}{\rho_0 L A(\theta)} \right], \quad (7.5)$$

where $\rho_0 = 1\text{M}$ is a reference concentration, and we highlighted the coupling with the geometry of the GUV via the θ -dependence. Note that, contrary to the case of two adhering GUVs [37], here $\Delta S_b^{\text{conf}} = \Delta S_\ell^{\text{conf}}$. The local roughness of the membranes could also influence ΔG [132] – this effects will be studied elsewhere.

We indicate the total number of a and a' tethers on the GUV with N , and model the SBL as an infinite reservoir of tethers. A combinatorial calculation detailed in the supplementary information allows the derivation of the overall hybridisation energy for the system of linkers

$$U_{\text{hyb}} = N k_B T \left[x_\ell + 2 \log(1 - x_\ell - x_b) - 2 \frac{\bar{N}_f}{N} \right], \quad (7.6)$$

In Eq. (7.6), x_b and x_ℓ are the fraction of tethers involved in bridges/loops, given by

$$\frac{x_b}{(1 - x_\ell - x_b)} = q_b, \quad (7.7)$$

$$\frac{x_\ell}{(1 - x_\ell - x_b)^2} = q_\ell \quad (7.8)$$

where

$$q_\ell = \exp[-\beta \Delta G^*], \quad (7.9)$$

$$q_b = \frac{\bar{N}_f}{N} \exp[-\beta \Delta G^*]. \quad (7.10)$$

In Eqs. (7.9) and (7.10) the hybridisation free energy is re-defined as $\Delta G^* = \Delta G - k_B T \log N$. The combinatorial contribution $-k_B T \log N$, typically estimated in $\sim -10 k_B T$, has a stabilising effect.

The quantity $\bar{N}_f$ appearing in Eq. (7.6) indicates the average number of unbound DNA tethers anchored to the SBL available within the adhesion patch. The concentration c_f of unbound tethers of each type (a or a'), such that $\bar{N}_f(\theta) = c_f A_p(\theta)$, is given by

$$c_0 - c_f = c_f^2 \frac{\exp[-\beta \Delta G_0]}{\rho_0 L}, \quad (7.11)$$

where $c_0 = \rho_{\text{DNA}}/2$ is the total concentration of a and a' tethers. The full derivation of Eq. (7.11) is provided in the supplementary information. Note that the concentration of loops on the SBL is $c_\ell = c_0 - c_f$.

Equation (7.6) generalises the expression found in ref. [37] to the case in which

vesicles are in contact with an infinite reservoir of thetethers. The overall DNA contribution to the interaction energy in Eq. (7.1) is

$$U_{\text{DNA}} = U_{\text{hyb}} - 2Nk_{\text{B}}T \log \left(\frac{A}{A_0} \right), \quad (7.12)$$

where the term on the right-hand side accounts for the change in overall confinement entropy following the area-change of the GUV [37].

Overall interaction energy.

By combining Eqs. (7.2), (7.6), and (7.12) into Eqs. (7.1), we obtain an analytical expression for the interaction energy of the GUV+SBL system as a function of the only independent variable θ

$$U(\theta) - U_0 = K_a \frac{[A(\theta) - \tilde{A}]^2}{2\tilde{A}} + Nk_{\text{B}}T \left[x_{\ell}(\theta) + 2 \log [1 - x_{\ell}(\theta) - x_{\text{b}}(\theta)] - 2 \frac{\tilde{N}_{\text{f}}(\theta)}{N} - 2 \log \left(\frac{A(\theta)}{A_0} \right) \right]. \quad (7.13)$$

Model parameters and error propagation

The model features seven input parameters: the thermal expansion coefficient α , the stretching modulus K_a , the length of the dsDNA tether L , the hybridisation enthalpy ΔH^0 and entropy ΔS^0 of the sticky ends, the DNA coating density ρ_{DNA} (used to calculate the overall number of strands per GUV – $2N$), the neutral temperature T_0 and radius R_0 .

The stretching modulus is estimated from literature data as $K_a = 240 \pm 90$ mN m⁻¹, with the error bar covering the entire range of reported values [133–136]. The thermal expansion coefficient has been experimentally estimated as $\alpha = 1.3 \pm 0.7$ K⁻¹ [37]. The hybridisation enthalpy and entropy are estimated according to the nearest neighbours thermodynamic rules [129] as $\Delta H^0 = -54 \pm 5$ kcal mol⁻¹ and $\Delta S^0 = -154 \pm 13$ cal mol⁻¹ K⁻¹. It is not clear whether the stabilising effect of the non-hybridised dangling bases neighbouring the sticky ends, in the present case the adenine bases present at both sides of the sequences (see FIG. 7.1), should be taken into account. It is indeed possible that their attractive contribution is compensated or overwhelmed by the repulsive effect of the long inert DNA connected to the sticky ends [130]. The errorbars in ΔH^0 and ΔS^0 are included to cover both these scenarios. The length $L = 9.8$ nm of the dsDNA spacers can be precisely estimated from the contour length of dsDNA (0.388 nm per base-pair [121]). The DNA coating density is estimated for different samples as explained in the experimental section. The neutral temperature T_0 changes widely from vesicle to vesicle due to the polydispersity of electroformed samples. We generally use T_0 as a fitting parameter. For a known T_0 , the neutral radius R_0 is experimentally determined as

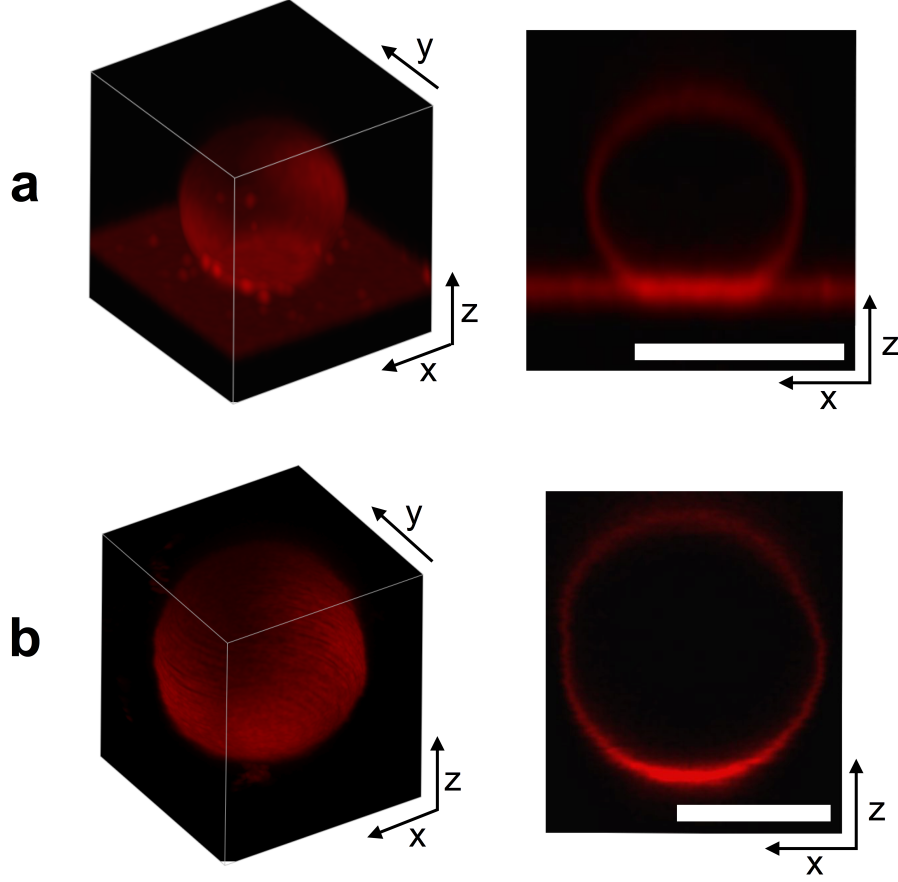


FIGURE 7.3: From left to right: 3D reconstruction from confocal z-stack, and confocal cross section of an adhering GUV (a) and a non-adhering GUV on a glass substrate (b). For 3D rendering, confocal images have been acquired with a Leica HCX PL APO CS 63×1.4 NA oil immersion objective for better resolution, and deconvolved using the experimental point-spread function. Scale bars: $10\ \mu\text{m}$.

$R_0 = R|_{T=T_0}$. If T_0 falls outside the experimentally accessible temperature range, we extrapolate

$$R_0 \approx \sqrt{\frac{A(T_1)}{\pi [1 + \alpha (T_1 - T_0)]}}, \quad (7.14)$$

where T_1 is the minimum experimentally accessible temperature.

Errors in the input parameters are numerically propagated to the theoretical predictions [37]. Briefly, we sample the results of the model using random values of the input parameters $X \pm \Delta X$ extracted from a Gaussian distribution with mean equal to X and standard deviation equal to ΔX . From a sample of size ≥ 10000 , we estimate the theoretical prediction of each observable as the median of the sampled distribution. Errorbars cover the interval between the 16th and the 84th percentile.

TABLE 7.1: Input parameters of the model

$\alpha = 1.3 \pm 0.7 \text{ K}^{-1}$
$K_a = 240 \pm 90 \text{ mN m}^{-1}$
$L = 9.8 \text{ nm}$
$\Delta H^0 = -54 \pm 5 \text{ kcal mol}^{-1}$
$\Delta S^0 = -154 \pm 13 \text{ cal mol}^{-1} \text{ K}^{-1}$

7.4 Results and discussion

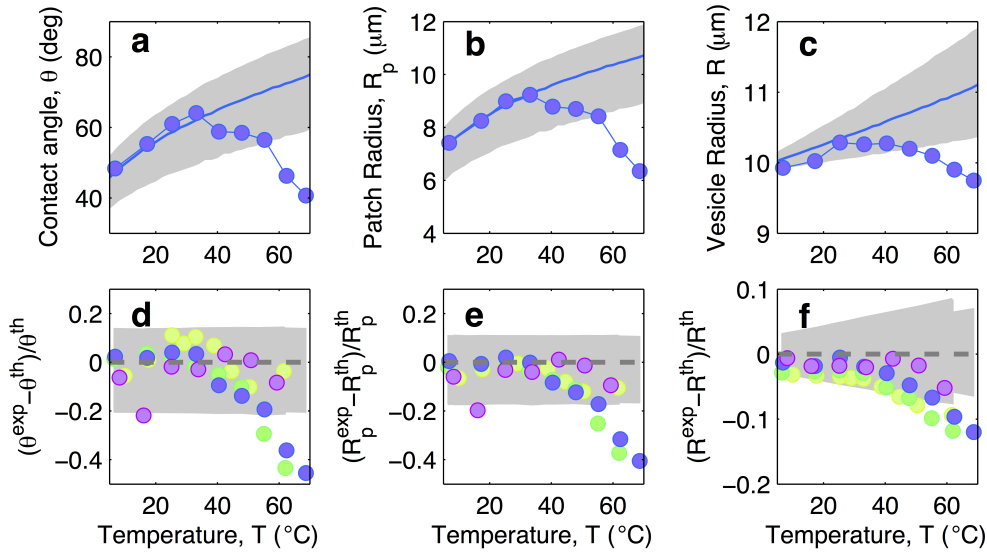


FIGURE 7.4: Experimental and theoretical temperature-dependent vesicle adhesion for samples with intermediate DNA coating density $\rho_{\text{DNA}}^{\text{med}} = 390 \pm 90 \mu\text{m}^{-2}$ (see experimental section). In the top row we demonstrate the temperature-dependence of the contact angle θ (a), adhesion-patch radius R_p (b), and vesicle radius R (c) for a typical adhering GUV. Points indicate experimental data, and solid lines mark theoretical predictions, with errorbars visualised as grey-shaded regions. Fitting parameter $T_0 = -40^\circ\text{C}$. In the bottom row we summarise the results for 5 vesicles. Points represent the relative deviation of experimental data from theoretical predictions $(X^{\text{exp}} - X^{\text{th}})/X^{\text{th}}$, with $X = \theta$ (d), R_p (e), and R (f). Grey-shaded regions mark the uncertainty interval of the theory. Model parameters are reported in TABLE 7.1.

Qualitative observations

In FIG. 7.3, we can visually compare confocal images of a DNA-functionalised GUV adhering to a SBL (a) and a non-adhering GUV on a passivated glass surface (b). Both GUVs and SBL are stained with fluorescent lipids.

It is clear from both the 3D reconstruction and the vertical cross section that the adhering GUV takes the shape of a truncated sphere, with a flat and circular contact region. The fluorescence intensity measured within the adhesion patches is almost exactly equal to twice the value measured on the SBL outside the adhesion region (1.95 times for the vesicle shown in FIG. 7.3 a). This evidence confirms the presence of two lipid bilayers in close contact within the adhesion area and excludes the possibility of DNA-mediated fusion of the two membranes [137].

The non-adhering GUV displayed in FIG. 7.3 b does not exhibit a flat adhesion patch, as clear from the vertical cross-section. Note that for both the adhering and the non-adhering GUVs, the bottom part of the stacks appears brighter due to the z -dependent response of the instrument.

Temperature-dependence of the geometrical observables

In this section we discuss the temperature-dependence of the morphology of adhering GUVs. In FIG. 7.4 a-c we show the experimentally determined contact angle θ , adhesion-patch radius R_p , and vesicle radius R for a typical adhering GUV in a sample with DNA concentration equal to $\rho_{\text{DNA}}^{\text{med}} = 390 \pm 90 \mu\text{m}^{-2}$. The contact angle displays a non-monotonic behaviour as a function of temperature, with a positive slope at low T , followed by a sudden decay for T higher than $\sim 30^\circ\text{C}$. The adhesion radius follows the same trend, as does the vesicle radius, which however displays much smaller relative variations. The solid lines in FIG. 7.4 a-c represent theoretical predictions calculated using the input parameters in TABLE 7.1, and using the neutral temperature T_0 as a fitting parameter. Grey-shaded regions indicate propagated uncertainty in the theoretical predictions. The agreement between theory and experiments is quantitative at low temperatures. At high T , the theory fails to predict the drop in contact angle observed in experiments. This behaviour is expected since our theoretical description is valid in the limit of strong adhesion, where the attractive forces are sufficient to suppress bending contributions in the interaction energy. At high temperature the DNA, which in the present experiment features relatively short sticky ends, starts to melt, causing the loosening of the adhesive forces and a change in the GUV shape, detected as a shrinkage of the adhesion area. The temperature-dependence of the fraction of DNA bonds is quantified and discussed in the following sections.

In FIG. 7.4 d-f we show the relative deviations of the experimentally-determined morphological observables from the theoretical predictions, defined as $(X^{\text{exp}} - X^{\text{th}})/X^{\text{th}}$, for $X = \theta$, A_p , and A . The data, collected from 5 vesicles, are consistent: the experimental data fall within theoretical errorbars at low T , deviating at higher temperature due to the failure of the strong adhesion assumption.

DNA-melting

We investigate the temperature-dependence of the fraction of formed DNA bonds via in-situ FRET measurements. Cyanine fluorophores, Cy3 (donor) and Cy5 (acceptor), are connected to the 3' termini of a and a' sticky ends, as sketched in

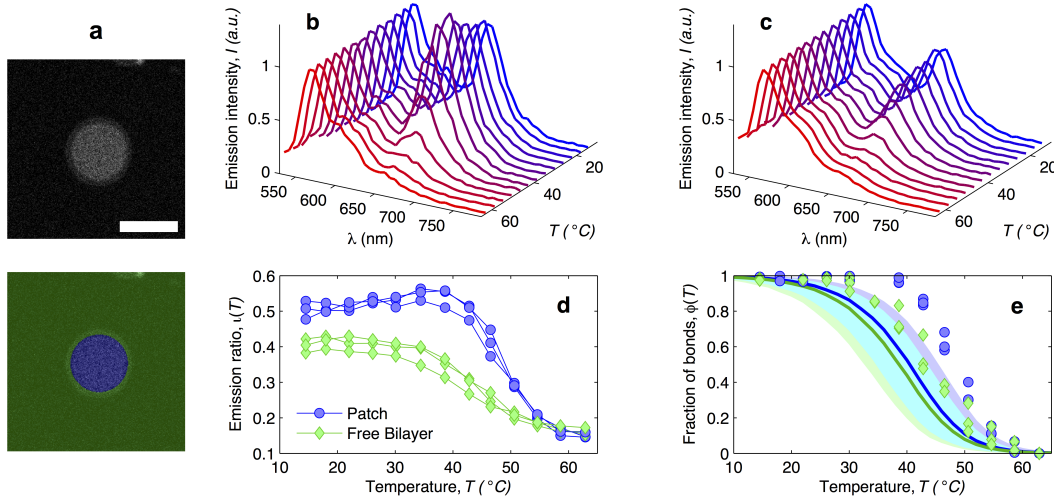


FIGURE 7.5: In-situ FRET spectroscopy characterisation of the temperature-dependence of the fraction of DNA bonds. **a**, Confocal image of the adhesion patch of a GUV. At the bottom we show the same image segmented with our software to separate the adhesion patch (blue) from the free supported bilayer (green). Scale bar: 15 μm . **b**, Fluorescence emission spectra measured within the adhesion patch for a typical vesicle (blue area in panel **a**). Colours from blue to red mark low to high temperatures in the interval $14.5 \leq T \leq 62.9$ °C. The curves are normalised to the emission peak of Cy3 (568nm). In panel **c**, we show the emission spectra measured on the free SBL (green area in panel **a**). **d**, Relative intensity of the Cy5 emission peak (665nm) measured within (blue circles) and outside (green lozenges) the adhesion patch. The curves are relative to 3 different vesicles. The amplitude of the Cy5 and Cy3 emission peaks is determined through a Gaussian fit of the 5 data points closest to the maximum. **e**, Experimental (symbols) and theoretically predicted (solid lines) fraction of formed DNA bonds within (blue circles) and outside (green lozenges) the adhesion patch. Experimental data are extracted from the curves in panel **d** as described in the text. Theoretical curves are calculated using the parameters in TABLE 7.1, DNA-coating density $\rho_{\text{DNA}}^{\text{high}} = 1200 \pm 300 \mu\text{m}^{-2}$, $T_0 = -20$, and $R_0 = 10 \mu\text{m}$. Note that the value of T_0 does not significantly affect these quantities. Blue and Green shaded regions indicate propagated uncertainties of the blue and green solid lines. Cyan shaded region marks their overlap.

FIG. 7.1. FRET efficiency is described by

$$E = \frac{1}{1 + \left(\frac{d}{R_0}\right)^6}, \quad (7.15)$$

where d is the distance between the fluorophores and R_0 is the Forster radius, equal to 5.4 nm for the case of Cy3-Cy5 [138]. When a pair of sticky ends are bound to form a loop or a bridge, the distance between Cy3 and Cy5 is approximately equal

to the length of the hybridised sticky ends – 2.7 nm, therefore we can assume a very high energy transfer efficiency for bound linkers. The average distance between unbound linkers is sufficiently long to guarantee a comparatively very low transfer efficiency between unpaired tethers. Note that for FRET experiments the lipid membrane is not stained with Texas Red to avoid spurious signal (energy transfer between Texas Red and Cy5). In FIG. 7.5 a, we show the confocal image of an adhesion patch (top), segmented to separate the actual adhesion area from the surrounding free SBL. This enables an efficient characterisation of FRET efficiency in-situ. The emission spectra, collected within the patch and on the SBL while exciting the donor at 514 nm, are shown in FIG. 7.5 b and c, respectively. Colours from blue to red indicate low to high temperature. Spectra are normalised to the emission peak of Cy3, correctly found at ~ 568 nm. Note that for this experiment we used high DNA concentration $\rho_{\text{DNA}}^{\text{high}}$ to strengthen the signal that otherwise would be too weak for a wavelength scan. As expected, the emission of the acceptor, peaked at ~ 665 nm, visibly drops at high temperature. We quantify this effect in FIG. 7.5 d, where we plot the normalised acceptor emission intensity $\iota = I_{\text{Cy5}} / (I_{\text{Cy5}} + I_{\text{Cy3}})$ [139], where the $I_{\text{Cy5/Cy3}}$ are the peak-intensities estimated through a local Gaussian fit. Although qualitatively similar, the ι curves measured within and outside the adhesion patch exhibit some differences. For the case of free SBL, the emission ratio remains constant (~ 0.4) or slightly decreases upon heating, up to $\sim 40^\circ\text{C}$, then it gradually drops down to ~ 0.15 . This decay is ascribed to the melting transition of DNA loops formed within the SBL. The FRET signal measured within the patch is higher at low temperatures (~ 0.6). This effect is probably due to the higher DNA density found in the patch at low temperature, which increases the probability of energy transfer between unpaired strands. In fact, we find that the overall fluorescence intensity measured within the patch at $T < 20^\circ\text{C}$ is between 6 and 13 times higher than the intensity measured on the SBL. The FRET signal measured within the patch is also found to increase upon heating, before suddenly decreasing at $T \sim 45^\circ\text{C}$. The increase in FRET efficiency cannot be explained, since the local DNA density decreases as the adhesion area becomes larger upon heating. At high temperatures, the FRET signal measured within the patch plateaus at ~ 0.15 , in line with what we measure on the SBL. This confirms that at high enough temperature, when no bonds are formed, the DNA concentration is uniform across all the surfaces.

The curves $\iota(T)$ can be used to semi-quantitatively estimate the temperature dependence of the overall fraction of DNA bonds. We fit the low temperature plateaus ($T < 35^\circ\text{C}$) in FIG. 7.5 d, with linear baselines $B(T)$ and assume that $\iota(T)$ plateaus to a constant value for $T > 57^\circ\text{C}$. The fraction of formed DNA bonds is thereby estimated as

$$\phi(T) = \frac{\iota(T) - \langle \iota_{T>57^\circ\text{C}} \rangle}{B(T) - \langle \iota_{T>57^\circ\text{C}} \rangle}. \quad (7.16)$$

A better estimate of $\phi(T)$ could be obtained by measuring $\iota(T)$ up to higher temperatures, and fitting the high-temperature plateau with a second linear baseline. However, temperatures higher than 65°C can not be safely probed due to the risk of destabilisation of the dsDNA spacers. The experimental $\phi(T)$ data in FIG. 7.5 e

indicate that the DNA melting transition is broad, spanning more than 30°C [140]. Moreover, the melting seems to occur at a higher temperature (by about 5°C) within the adhesion patch.

The experimentally estimated fraction of DNA bonds can be compared with theoretical predictions. On the free SBL, only loops can form, and therefore $\phi(T)$ should be compared to the fraction of loops x_ℓ^{SBL} , calculated according to Eqs. (7.11), (7.38)-(7.39). Within the adhesion patch we count contributions from bridges, loops formed on the GUV, and loops formed on the SBL. By assuming evenly distributed loops, the overall number of DNA bonds found within the patch is

$$N_{\text{bound}} = N \left(\frac{A_p}{A} x_\ell + x_b \right) + c_0 x_\ell^{\text{SBL}} A_p, \quad (7.17)$$

where $x_{b/\ell}$ are the fractions of loops and bridges on the GUV, given by Eqs. (7.48) and (7.49). The overall number of DNA tethers, bound and unbound, found within the patch is

$$N_{\text{tot}} = N \left[\frac{A_p}{A} (1 - x_b) + x_b \right] + c_0 A_p, \quad (7.18)$$

where we assume that also unbound DNA is evenly distributed across the surfaces. The theoretically predicted fraction of bonds within the patch is thus $N_{\text{bound}}/N_{\text{tot}}$. In FIG. 7.5 e we compare theoretical $\phi(T)$ with experimental estimates. Since the choice of the neutral temperature T_0 does not noticeably affect the melting curves, theoretical predictions are calculated using a fixed $T_0 = -20^\circ\text{C}$ and $R_0 = 10 \mu\text{m}$. Our model captures the width of the DNA transition as well as the difference in melting temperature between the patch and the free SBL. However, the theory underestimates the average melting point by 5 – 10°C. This deviation is, at least partially, ascribable to the attractive effect of Cy3 and Cy5 molecules on the stability of DNA. For duplexes labeled with either of the molecules, the stabilisation has been quantified in a positive melting-temperature shift of 1.4 – 1.5°C [141]. The presence of both molecules is expected to cause a greater shift. The impossibility of probing the high-temperature baselines could also play a role.

Membrane tension

The temperature dependence of the membrane tension is measured by flickering analysis of the equatorial cross sections of GUVs. The tension is extracted by fitting the power spectra of the thermal fluctuations with the function

$$\langle h(q_x)^2 \rangle = \frac{1}{L} \frac{k_B T}{2\sigma} \left[\frac{1}{q_x} - \frac{1}{\sqrt{q_x^2 + \frac{\sigma}{\kappa}}} \right], \quad (7.19)$$

where L is the contour length of the equatorial cross-section of the GUV, σ is the membrane tension, κ is the bending modulus, and q_x is the wave vector evaluated along the contour. Equation (7.19) is derived from the original work of Helfrich [120], describing the fluctuations of an infinite 2D membrane, and corrected to account for the fact that, by imaging an equatorial cross-sections, only modes

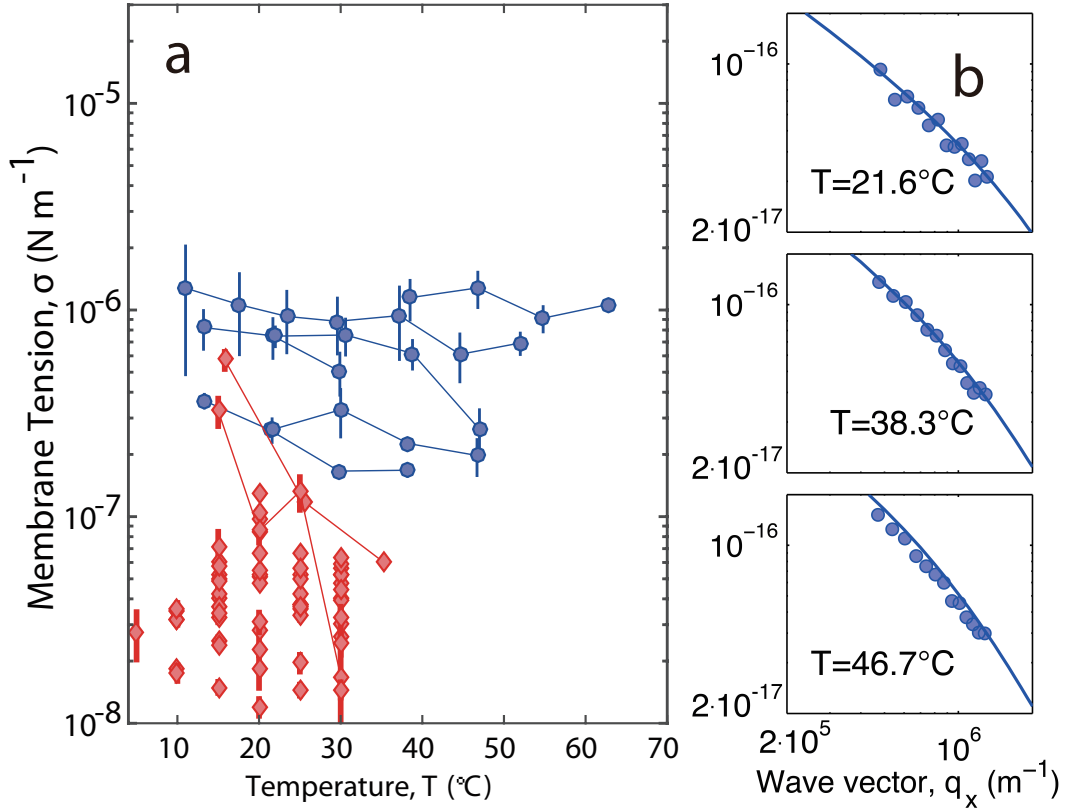


FIGURE 7.6: Experimental temperature-dependent membrane tension. **a**, Membrane tension σ measured from flickering experiments. Blue circles indicate adhering GUVs with low DNA coverage ($\rho_{\text{DNA}}^{\text{low}} = 39 \pm 9 \mu\text{m}^{-2}$). Red lozenges indicate non-adhering GUVs on a BSA-coated glass surface. **b**, Fluctuation-amplitude spectra for adhering GUVs with low DNA coverage and various temperatures. Symbols indicate experimental data and solid lines indicate fits according to Eq. (7.19). From top to bottom the fitted values of the membrane tension are $\sigma = 4.1 \pm 0.5, 2.42 \pm 0.09, \text{ and } 1.88 \pm 0.09 \times 10^{-7} \text{ Nm}^{-1}$.

propagating along the horizontal direction should be considered [88]. Of the discrete set of wave vectors $q_x(n) = 2\pi n/L$, modes with $n < 6$ are excluded from the analysis. Mode $n = 0$ and $n = 1$ correspond to size changes and translations of the GUV. Modes with $n > 2$ describe thermal fluctuations. However, Eq. (7.19) is derived for a planar membrane, and should not be used to describe modes with $n < 6$, which are influenced by the spherical geometry of the GUV [88]. For our analysis, we fit the spectra for modes $6 \leq n \leq 16$. At higher q we approach the resolution limits of the current method.

In FIG. 7.6 a, blue circles mark the tension measured as a function of temperature for adhering GUVs. In FIG. 7.6 b, we show examples of power spectra fitted by Eq. (7.19). The tension typically lies in the interval $2 \times 10^{-7} - 2 \times 10^{-6} \text{ N m}^{-1}$, with clear variations between different GUVs. In the tested range, the tension consistently displays a weak dependence on temperature changes.

For comparison, the membrane tension is measured on non-adhering GUVs

supported by a passivated glass substrate. The values of σ measured for non-adhering GUVs (red lozenges in FIG. 7.6 a) are significantly lower than those measured on adhering vesicles, falling within the range $10^{-9} - 10^{-6} \text{ N m}^{-1}$, and being clustered around 10^{-8} N m^{-1} . Furthermore, membrane tension of non-adhering GUVs typically displays a strong decrease upon increasing temperature. The large variability observed in the tension of adhering and, in particular, of free GUVs, is ascribed to the polydispersity of electroformed samples, which produces vesicle populations with very different excess areas (T_0).

With the present technique, we can not access the tension of vesicles adhering to SBL for the case of higher DNA concentrations. Indeed, for values of $\sigma > 6 \times 10^{-6} (\text{Nm}^{-1})$, the portions of the fluctuation power spectra are masked by experimental noise deriving from the finite resolution of the contour-tracking procedure.

The membrane tension can be evaluated within the framework of our model. At equilibrium, the derivative of the interaction energy in Eq. (7.1), taken with respect to the GUV area, is

$$\frac{\partial U}{\partial A} = \sigma + \frac{\partial U_{\text{DNA}}}{\partial A} = 0, \quad (7.20)$$

where we used

$$\sigma = \frac{\partial U_{\text{stretching}}}{\partial A} = K_a \frac{A - \tilde{A}}{\tilde{A}}. \quad (7.21)$$

Equation (7.20) suggests that by measuring σ we can directly probe the DNA-mediated forces per unit length. However, we could not access the tension regime, where the strong adhesion limit is reasonable, using by flickering analysis. Note that we reduced the DNA concentration to measure the surface tension. Further investigation of the membrane tension by micro pipette aspiration methodology would reveal the force generated by DNA tethers.

7.5 Summary

In this part, we experimentally investigated temperature-dependent adhesion of Giant-Unilamellar-Vesicles on supported lipid bilayers mediated by mobile DNA linkers. The simple geometry of the problem allowed for an accurate characterisation of the morphology of adhering GUVs and the temperature dependent fraction of bound DNA tethers by means of confocal microscopy. For the first time to our knowledge, we quantified the temperature-dependent membrane tension induced by DNA bonds by analyzing the thermal fluctuations of the GUVs imaged across their equatorial plane.

Some parts of the experimental results were compared to theoretical predictions from the recently developed model [37], which we here extended to the case of vesicle-plane adhesion. The model took into account both the elastic deformation of the GUV and the statistical-mechanical details of the DNA-mediated interactions.

For sufficiently high DNA coverage, the adhesion contact angle exhibited a re-entrant temperature dependence. Upon heating from low temperature the contact

angle increased, reaching a maximum at $T \simeq 30 - 40^\circ\text{C}$. Upon further temperature increase, the contact angle dropped. With a single fitting parameter, the model was capable of quantitatively predicting the low temperature regime and ascribes the increase in contact angle to the interplay between the temperature-dependent excess area of the GUV and the entropic coupling between the hybridisation free-energy of the mobile tethers and the adhesion area. The theory was developed in the limit of strong adhesion, therefore it failed to predict the re-entrant behaviour of the adhesion area, caused by the weakening of the DNA bonds.

The melting of DNA bonds was investigated in-situ by FRET measurements. We observed a broad melting transition and found that bonds formed within the GUV-plane adhesion patch were more stable than in-plane bonds formed on free bilayers. Our model could semi-quantitatively reproduce these features, although an underestimation of the melting temperature was observed.

Membrane tension measurements performed on adhering GUVs demonstrated a weak temperature dependence. In a similar range of temperatures, non-adhering GUVs exhibited significantly lower tension, rapidly decreasing upon heating. The differences in magnitude and trend demonstrated the role played by DNA in mediating membrane adhesion.

7.6 Supplementary information

Details on model derivation

Geometrical expressions

In the limit of strong adhesion the GUV will take the shape of a truncated sphere with contact angle θ (FIG. 7.1), which we take as the independent variable of our model [126]. In this simple geometry, the contact (patch) area, total area, and volume of the GUV are respectively

$$A_p = \pi R^2 \sin^2 \theta \quad (7.22)$$

$$A = \pi R^2 (1 + \cos \theta) (3 - \cos \theta) \quad (7.23)$$

$$V = \frac{\pi R^3}{3} (1 + \cos \theta)^2 (2 - \cos \theta). \quad (7.24)$$

In the limit of water-impermeable membranes, the internal volume of the GUVs can be taken as a constant

$$V = \frac{4}{3} \pi R_0^3, \quad (7.25)$$

where we introduce a reference radius R_0 . By using Eqs. (7.24) and (7.25) we obtain

$$R = R_0 \left[\frac{4}{(1 + \cos \theta)^2 (2 - \cos \theta)} \right]^{1/3}, \quad (7.26)$$

which can be inserted into Eqs. (7.22) and (7.23) to make the θ -dependence of A_p and A explicit.

Let us now recall the definition of reduced volume of a vesicle [126, 142]

$$v = \frac{\frac{3V}{4\pi}}{\left(\frac{\tilde{A}}{4\pi}\right)^{3/2}}. \quad (7.27)$$

By combining Eq. (7.27) with expression for the temperature-dependent unstretched area in Eq. (7.3), we obtain

$$v = [1 + \alpha(T - T_0)]^{-3/2}. \quad (7.28)$$

The reference temperature T_0 is therefore defined as the temperature at which a GUV has reduced volume equal to 1. For $T < T_0$, when $v > 1$, an isolated vesicle resembles a turgid sphere, with non-zero membrane tension whereas for $T > T_0$, $v < 1$, it assumes the features of a “floppy” balloon, with excess area. At $T = T_0$ an isolated vesicle is a perfect sphere with zero-membrane tension and radius equal to the reference radius R_0 .

By combining Eqs. (7.23), (7.26) and (7.26) we obtain an explicit, θ -dependent expression for the stretching energy in Eq. 7.2. Note that Eq. (7.26) has been derived under a constant-volume assumption (Eq. 7.25). Alternatively, an equivalent relation can be derived for water permeable – solute impermeable – GUVs, in which the volume is set by the balance between the osmotic pressure drop across the membrane and the Laplace pressure [126]. In relevant experimental conditions the two assumptions lead to very similar results [37].

Free energy for bridge and loop formation.

For the case of mobile linkers, the configurational entropic contribution to the bridge/loop formation free energy $\Delta S_{b/\ell}^{\text{conf}}$ (Eq. 7.4) can be split into a rotational and translational contribution

$$\Delta S_{b/\ell}^{\text{conf}} = \Delta S^{\text{rot}} + \Delta S_{b/\ell}^{\text{trans}}. \quad (7.29)$$

The rotational contribution takes the same expression for loop and bridge formation, and encodes for the reduction of configurational entropy following the hybridisation of two rigid tethers with fixed grafting sites [37, 110]

$$\Delta S^{\text{rot}} = k_B \log \left[\frac{1}{4\pi\rho_0 L^3} \right], \quad (7.30)$$

where $\rho_0 = 1\text{M}$ is a reference concentration. The translational contribution encodes for the lateral confinement following the binding of two mobile tethers. For the case of loops, upon binding, two tethers initially capable of exploring the entire GUV surface area A , are confined to within a region $\sim L^2$ from each other [37]

$$\Delta S_{\ell}^{\text{trans}} = k_B \log \left[\frac{4\pi L^2 A}{A^2} \right] = k_B \log \left[\frac{4\pi L^2}{A} \right]. \quad (7.31)$$

For the case of bridge formation, we consider a free linker on the SBL, initially located within the contact area A_p , and a second linker on the GUV, which is free to explore the entire surface area A . Upon binding, the area available to the pair is reduced to $4\pi L^2 A_p$, resulting in

$$\Delta S_b^{\text{trans}} = k_B \log \left[\frac{4\pi L^2 A_p}{A A_p} \right] = k_B \log \left[\frac{4\pi L^2}{A} \right]. \quad (7.32)$$

We notice that, contrary to the case of two adhering GUVs [37] here $\Delta S_b^{\text{trans}} = \Delta S_\ell^{\text{trans}}$. By combining Eqs. (7.29)–(7.32) with Eq. (7.4), we obtain the hybridisation free energy of bridge formation on the loops formation on the GUV in Eq. (7.5).

Fraction of loops and free tethers on the SBL.

We now focus on the description of the tethers anchored to the SBL, and calculate the equilibrium fraction of formed loops in the absence of an adhering GUV. This information is needed for the calculation of the GUV-SBL adhesive interaction as well as for a direct comparison with experimental data.

Let us consider a finite portion of the SBL of area Σ , containing two populations of N linkers with a and a' sticky ends. Following Eq. (7.5), the free energy for loop (ℓ) formation on the SBL can be written as

$$\Delta G_\ell^{\text{SBL}} = \Delta G^0 - k_B T \log \left[\frac{1}{\rho_0 L \Sigma} \right]. \quad (7.33)$$

By indicating as N_ℓ the number of loops within the SBL, and taking into account combinatorics, we can write the partition function of this systems as [37]

$$Z = \sum_{N_\ell} \binom{N}{N_\ell}^2 N_\ell! \exp(-\beta N_\ell \Delta G_\ell^{\text{SBL}}), \quad (7.34)$$

which can be rearranged as

$$Z = \sum_{N_\ell} e^{-S(N_\ell)}. \quad (7.35)$$

We now consider the limit of an infinite SBL with a constant DNA surface density c_0 , i.e. we take $N, \Sigma \rightarrow \infty$, with $c_0 = N/\Sigma = \rho_{\text{DNA}}/2$. By using the Stirling approximation we obtain

$$S(c_\ell) = \text{const} \cdot \Sigma [-c_\ell \log c_\ell - 2(c_0 - c_\ell) \log(c_0 - c_\ell) - c_\ell \beta \Delta G^0 - c_\ell \log(\rho_0 L) - c_\ell + \text{const}], \quad (7.36)$$

where we define the density of loops as $c_\ell = N_\ell/\Sigma$. Within the saddle-point approximation [37], the sum in Eq. (7.35) is dominated by the stationary point of S

$$\frac{\partial S}{\partial c_\ell} = 0. \quad (7.37)$$

By solving Eq. (7.37) we obtain the expression in Eq. (7.11) in the text, where we introduce the concentration of free tethers on the SBL $c_f = c_0 - c_\ell$. Note that with Eq. (7.11) we recover a simple mass-balance relation between loops and free tethers on the SBL, which ultimately results in

$$c_\ell = c_0 \frac{2q_{\text{SBL}} + 1 - \sqrt{4q_{\text{SBL}} + 1}}{2q_{\text{SBL}}}, \quad (7.38)$$

where

$$q_{\text{SBL}} = \frac{c_0}{\rho_0 L} \exp[-\beta \Delta G_0]. \quad (7.39)$$

The fraction of loops formed within the bilayer is $x_\ell^{\text{SBL}} = c_\ell / c_0$.

Combinatorial effects.

Given the expressions for the hybridisation free-energy of a single bridge/loop (Eq. (7.5)), a combinatorial approach is required to compute the overall DNA-mediated interaction energy.

Following the derivation carried out to describe loop formation on the SBL, we indicate the total number of tethers with a (a') sticky ends on the GUV as N , and define N_ℓ as the number of those tethers linked in loops. We indicate as N_{bi} , with $i = 1, 2$, the number of tethers forming bridges with those on the SBL, with the index i referring to a and a' sticky ends. We label as N_{fi} the number of free tethers on the SBL located within the adhesion patch, where the index $i = 1, 2$ now refers to a' and a sticky ends. The partition function of the system of linkers can be written as

$$z(N_{f1}, N_{f2}, N) = \sum_{N_{bi}, N_\ell} [\Omega_{N_{f1}, N_{f2}, N}(N_{b1}, N_{b2}, N_\ell) \exp[-\beta(N_{b1} + N_{b2} + N_\ell)\Delta G]],$$

where the number of possible configurations for a given N_ℓ and N_{bi} is

$$\Omega_{N_{f1}, N_{f2}, N}(N_{b1}, N_{b2}, N_\ell) = N_\ell! \prod_{i=1,2} \binom{N_{fi}}{N_{bi}} \binom{N}{N_{bi}} \binom{N - N_{bi}}{N_\ell} N_{bi}! \quad (7.40)$$

To account for strand-concentration fluctuations within the adhesion patch, we need to consider that N_{fi} is Poisson-distributed around its average value $\bar{N}_f$

$$P(N_{fi}, \bar{N}_f) = \exp[-\bar{N}_f] \frac{\bar{N}_f^{N_{fi}}}{N_{fi}!}. \quad (7.41)$$

Using Eq. (7.38), and recalling that $c_f = c_0 - c_\ell$ is the concentration of free tethers within the SBL, we find

$$\bar{N}_f(\theta) = c_f A_p(\theta), \quad (7.42)$$

where we highlighted the strong dependence on the contact angle θ . Using Eqs. (7.40) and (7.41) we write the full partition function as

$$Z_{N_{f1}, N_{f2}}(\bar{N}_f, N) = \sum_{N_f} P(N_{f1}, \bar{N}_f) P(N_{f2}, \bar{N}_f) z(N_{f1}, N_{f1}, N). \quad (7.43)$$

Equation (7.43) can be rearranged as

$$Z_{N_{f1}, N_{f2}}(\bar{N}_f, N) = \sum_{N_{f1}, N_{f2}, N_\ell, N_{b1}, N_{b2}} \exp[-N\mathcal{A}]. \quad (7.44)$$

By defining the fractions $x_y = N_y/N$ ($y = b1, b2, l, f1, f2$), and using the Stirling approximation, $\mathcal{A}$ can be expressed as

$$\begin{aligned} \mathcal{A} = & \beta\Delta G^* x_\ell + x_\ell (\log x_\ell + 1) + \sum_{i=1,2} \left[\beta\Delta G^* x_{bi} + x_{fi} (\log N - \log \bar{N}_f - 1) + \right. \\ & \left. x_{bi} (\log x_{bi} + 1) + (x_{fi} - x_{bi}) \log (x_{fi} - x_{bi}) + (1 - x_\ell - x_{bi}) \log (1 - x_\ell - x_{bi}) \right]. \end{aligned} \quad (7.45)$$

Note that in Eq. (7.45) we re-defined the hybridisation free energy for bridge and loop formation as

$$\Delta G^* = \Delta G - k_B T \log N. \quad (7.46)$$

For typical experimental conditions, the attractive combinatorial term $-k_B T \log N$ in Eq. (7.46) can be estimated in $\approx -10k_B T$ [37]. Within the saddle-point approximation, the sum in Eq. (7.44) is dominated by the stationary point of $\mathcal{A}$

$$\frac{\partial \mathcal{A}}{\partial x_y} = 0 \text{ with } y=b1, b2, l, f1, f2. \quad (7.47)$$

From the saddle-point equations Eq. (7.47) we obtain Eqs. (7.7) and (7.8) in the text, where we find $x_{b1} = x_{b2} = x_b$. By solving Eqs. (7.7) and (7.8) we obtain

$$x_b = \frac{q_b \left(\sqrt{q_b^2 + 2q_b + 4q_\ell + 1} - q_b - 1 \right)}{2q_\ell} \quad (7.48)$$

$$x_\ell = \frac{q_b^2 + 2q_b + 2q_\ell + 1 - (q_b + 1) \sqrt{q_b^2 + 2q_b + 4q_\ell + 1}}{2q_\ell}. \quad (7.49)$$

Note that for simplicity the fraction of bridges and loops are indicated as $x_{b/\ell}$. The saddle point equations for x_f ($x_{f,1} = x_{f,2}$) read $x_f - x_b = \bar{N}_f/N$, which confirms that the density of the free tethers in the patch region is equal to that of the reservoir, as expected.

By inserting the saddle-point solutions for x_ℓ , x_b , and x_f in Eqs. (7.44) and (7.45) we can calculate the free energy U_{hyb} (Eq. (7.6)) [107].

Reference energy.

The reference energy U_0 in Eq.(7.1) is calculated for isolated GUV and SBL and can be written as

$$U_0 = U_0^{\text{stretching}} + U_0^{\text{DNA}}. \quad (7.50)$$

The stretching term is [37]

$$U_0^{\text{stretching}} = \begin{cases} 0 & \text{if } T \geq T_0 \\ K_a \frac{(A_0 - \tilde{A})^2}{A_0} & \text{if } T < T_0. \end{cases} \quad (7.51)$$

Note that the stretching contribution is only present for pre-stretched vesicles, i.e. if the reduced volume is $v > 1$ (i.e. $T < T_0$) [126]. The DNA contribution is calculated for a GUV of area equal to the unstretched area $\tilde{A}$, in which only loops can form. By following the steps outlined in section 7.6 and in ref. [37], we calculate the fraction of tethers involved in loops

$$x_\ell^0 = \frac{2q_\ell + 1 - \sqrt{4q_\ell + 1}}{2q_\ell}, \quad (7.52)$$

where q_ℓ is given by Eq. (7.9). The DNA part of the reference energy is

$$U_0^{\text{DNA}} = Nk_B T \left[x_\ell^0 + 2 \log(1 - x_\ell^0) - 2 \frac{\tilde{N}_f}{N} - 2 \log\left(\frac{\tilde{A}}{A_0}\right) \right], \quad (7.53)$$

where $\tilde{N}_f = c_f \tilde{A}_p$ is the number of free tethers present within area $\tilde{A}_p$ on the SBL. $\tilde{A}_p$ is the zero-stretching adhesion area, which the GUV-SBL system would form for negligibly small attractive forces when $T > T_0$, as derived in ref. [37]. Note that U_0 does not depend on the contact angle θ therefore its form does not influence the equilibrium features of the system.

Part IV

General conclusion

Chapter 8

Conclusion

8.1 Conclusion

In this thesis, the experimental, theoretical, and numerical results on dynamics of adhesive functionalized lipid bilayers are demonstrated. The main topics are divided into two parts (Parts II and III).

In Part II, we demonstrate morphological transitions from macro- to micro-phase separation in multi-component asymmetric lipid vesicles functionalized by glycolipids (GM1: monosialotetrahexosylganglioside), which are extracted by natural cells. In Chapter 3, for the first time to our knowledge, transition dynamics from macro- to micro-phase separation are experimentally revealed through confocal microscopy. The transitions occur via stripe-phase as a metastable state and finally lead to the emergence of size-monodisperse micro domains. In Chapter 4, to clarify the essential factors to drive the transitions, the transitions from macro- to micro-phase separation are numerically investigated using by the time-dependent Ginzburg-Landau equation to describe bending elastic membrane and phase separation. The findings give us some insight into the mechanism of the novel transitions, which are apparently governed by the local spontaneous curvature due to the local asymmetric lipid composition induced by externally added glycolipids (GM1). In Chapter 5, we demonstrate the effect of inserted GM1 into lipid bilayers on the bending modulus, to confirm the validity of the significant assumption in the numerical analysis. It is revealed that the bending moduli do not depend on the inserted GM1 in the tested range, guaranteeing the validity of the important assumption. In Chapter 6, we quantitatively reveal the negative relationship between the degree of lipid asymmetry and micro domain size in both of the experiments and numerical analysis, as predicted by our findings in Chapters 3–5.

The position of our findings in this part is represented in line with past studies. Firstly, in terms of biology, we describe scientific significance of our findings. Approximately four decades ago, J. Rothman revealed that the lipid composition in cell membranes is essentially asymmetric [77]. It is considered that the asymmetric distribution has biological importance, as glycolipids only exist in the outer layers for interacting with the other glycolipids and/or proteins. Meanwhile, the existence of nano-sized dynamic micro domains, enriched in saturated lipids, cholesterol, and proteins, has been pointed out for the last two decades [30]. In spite of the hectic investigations on the structures and functions, some problems have been unsolved so far. One unrevealed issue is the discrepancy of the micro

domain size in between *in-vivo* and reconstituted systems [18, 89]. Although it is considered that the micro domain in cells is nano-sized, micrometer-sized domains are readily reconstituted in GUVs. So far, the effect of the lipid asymmetry on phase separation has not been focused as the solution to the problem. Our findings in this part could provide important mechanistic clues for the long-standing unsolved problem, focused on the lipid asymmetry which is ubiquitous feature in cells [77]. The findings suggest that the degree of lipid asymmetry, physically meaning the magnitude of spontaneous curvature, could tune the micro domain size in reconstituted multi-component vesicles. Thus, we could build up a new hypothesis that cells control and maintain the micro domain size, named as lipid raft, by tuning the degree of *lipid asymmetry*. Secondly, in terms of physics, we describe scientific significance of our findings. The coarsening dynamics of phase separation have been intensively researched [19, 20, 47–53]. However, the transition dynamics from macro- to micro-phase separation has been poorly understood although it could occur in various systems if there exists any parameter to overcome the interface energy between two distinct phases. For the first time to our knowledge, the transition dynamics from macro- to micro-phase separation is revealed. In our system, long-range repulsive interaction via membrane curvature is induced by the locally-determined spontaneous curvature due to local asymmetric lipid composition.

In Part III, we experimentally investigate temperature-dependent adhesion of Giant-Unilamellar-Vesicles on supported lipid bilayers mediated by mobile DNA linkers. The simple geometry of the problem allows for an accurate characterisation of the morphology of adhering GUVs and the temperature dependent fraction of bound DNA tethers by means of confocal microscopy. For the first time to our knowledge, we quantify the temperature-dependent membrane tension induced by DNA bonds by analyzing the thermal fluctuations of the GUVs imaged across their equatorial plane.

The position of our findings in this part is described in accordance with preceding studies. Firstly, in terms of biology, we describe scientific significance of our findings. We found that the membrane tension is kept against the large temperature changes by adhesion due to multivalent interactions, in contrast to suspended lipid vesicles. The findings suggest that the membrane tension of cells is maintained through adhesion, which should relate to biological functions [35, 143], and that the estimation of mechanical parameters is essentially significant toward understanding cell mechanics using model systems adherent to other materials. Furthermore, the conclusions drawn for our model system can be adapted to the quantitative description of cell adhesion and spreading on solid substrates [144–148]. Secondly, in terms of material science, we describe scientific significance of our findings. Liposomes can transport substances into cells because of the stability in solution as well as other vesicular materials, e.g. nanogels [149], polymersomes [150], and Fullerene-based capsules [151]. So far, numerous materials toward pharmaceutical applications, including drug delivery and gene therapy, have been designed, greatly based on the fundamental understanding on soft matter physics [149–151]. However, the functionalization of vesicular materials has been still limited [37, 152, 153]. Our findings on functionalized liposome system

by mobile linkers can help the design of not only selective and responsive materials but also a new class of materials, namely tissue-like materials, with promised applications in biosensing, encapsulation-release mechanisms, and filtration [154].

To summarize, our findings in this thesis not only suggest that the functionalization of lipid bilayers to mimic cell membrane functions greatly changes the physical properties of lipid bilayers, but also give us some insight into biological phenomena, which has not been unveiled in the conventional studies on non-functionalized liposomes. The studies on functionalized liposome system have just barely began. The characteristic features, such as fluidity and large degree of freedom, give us a rich variety of intriguing phenomena in complex and soft system. Our findings in the thesis would contribute to the developing new field.

8.2 Future problems

In proceeding the studies on dynamics of adhesive functionalized lipid bilayers, many problems and difficulties have come out and some of them have remained to be unsolved. In this section, we introduce the unsolved future problems as follows.

In Part II, one of the intriguing tasks is to understand how long-wavelength fluctuations grow in the flat interface between two phases, e.g. the time dependence of the fluctuation amplitude spectra for each wave vector, and how they depend on the degree of lipid asymmetry, which should be a key parameter in determining the dynamics according to our findings. The important point would be the establishment of the simplified geometries, e.g. the lipid bilayer system supported by polymer-cushioned glass, because the facts that lipid vesicles are curved and suspended make it difficult to investigate them. It would be a key point to maintain the fluidity of lipids. A second theme may be the unsolved discrepancies on transition dynamics between the experimental and numerical results. The main inconsistent point is that macro-phase transits to micro-phase via stripe-micro phase in numerical analysis though the macro-phase transits to the micro-phase via stripe-phase as a metastable state in the experiments. Although various possibilities are considered, one of the fruitful challenges in the future would be to reveal the hydrodynamic effect on the transition dynamics using model H [48]. One of the other challenges might be to reveal the contribution of noise strength and/or the magnitude of line tension. Because of the heavy computational cost, the numerical investigations are still limited. The further comprehensive investigations might solve the problem.

In Part III, one of the significant tasks would be to prove the force generated by DNAs. In this part, the DNA concentration was reduced to weaken the force by DNA because of the accessible threshold of surface tension by flickering analysis with the present resolution. In this case, the strong adhesion limit would not be reasonable, as introduced in Chapter 2. Other methodologies, e.g. micro-pipette aspiration, should be useful in the higher surface tension regime, enabling us to directly prove the force generated by DNA because the strong adhesion limit can be safely assumed. In such cases, the experimental protocols established in this thesis

should also be applicable to the measurements. Furthermore, taking a broader perspective, one of the next ambitious and interesting challenges would be to understand the physical principles underlying a cluster of GUVs linked by sticky DNA. For example, revealing how the external force is transmitted into the tissue-like materials and the frequency characteristics is likely to give suggestive insights into the physics of tissue.

Appendix A

Mean and Gauss curvatures

The mean curvature H and Gauss curvature K are written by the two principal curvatures. The maximum and minimum values of possible curvatures, κ_1 and κ_2 , at a point (u_1, u_2) of a surface are the eigenvalues of the metric tensor h_{ij}^i , which are referred to as the two principal curvatures. Moreover, their eigenvectors are the principal vectors. Therefore, the two principal curvatures are the solutions of the following equation:

$$\left| \begin{pmatrix} L & M \\ M & N \end{pmatrix} - \kappa \begin{pmatrix} E & F \\ F & G \end{pmatrix} \right| = \kappa^2(EG - F^2) - \kappa(EN + GL - 2FM) + (LN - M^2) = 0. \quad (\text{A.1})$$

Using the two solutions, κ_α and κ_β , the mean curvature $H = (\kappa_\alpha + \kappa_\beta)/2$ and Gauss curvature $K = \kappa_\alpha \kappa_\beta$ are given by:

$$H = \frac{EN + GL - 2FM}{2(EG - F^2)},$$

$$K = \frac{LN - M^2}{EG - F^2}. \quad (\text{A.2})$$

If the two eigenvalues, κ_α and κ_β , are different, the two principal directions are orthogonal. The detailed proof is described below. We consider a unit vector $\vec{a} = l\vec{r}_{u_1} + m\vec{r}_{u_2}$ defined at the point (u_1, u_2) . The parameter set (l, m) of the two eigenvectors satisfies the following equation:

$$(EM - FL)l^2 + (EN - GL)lm + (FN - GM)m^2 = 0. \quad (\text{A.3})$$

When the two eigenvalues, κ_α and κ_β , are different, we can obtain the two solutions $u_\alpha = l_\alpha/m_\alpha$ and $u_\beta = l_\beta/m_\beta$. Using the fundamental forms, the sum and product of u_α and u_β are given by:

$$u_\alpha + u_\beta = -\frac{EN - GL}{EM - FL},$$

$$u_\alpha u_\beta = \frac{FN - GM}{EM - FL}. \quad (\text{A.4})$$

The inner product of the two unit vectors, $\vec{a}_\alpha = m_\alpha(\frac{l_\alpha}{m_\alpha}\vec{r}_{u_1} + \vec{r}_{u_2})$ and $\vec{a}_\beta = m_\beta(\frac{l_\beta}{m_\beta}\vec{r}_{u_1} + \vec{r}_{u_2})$ is given by:

$$\begin{aligned}
 \vec{a}_\alpha \cdot \vec{a}_\beta &= m_\alpha m_\beta (u_\alpha u_\beta \vec{r}_{u_1}^2 + (u_\alpha + u_\beta) \vec{r}_{u_1} \vec{r}_{u_2} + \vec{r}_{u_2}^2), \\
 &= m_\alpha m_\beta \left(\frac{FN - GM}{EM - FL} E - \frac{EN - GL}{EM - FL} F + G \right), \\
 &= m_\alpha m_\beta \frac{(FN - GM)E - (EN - GL)F + (EM - FL)G}{EM - FL}, \\
 &= 0
 \end{aligned} \tag{A.5}$$

Appendix B

Line tension measurements by flickering analysis

Flickering analysis is applicable to measure the line tension of the interface between two liquid phases. First, we consider a domain with a perimeter L . Then, If there are fluctuations, the increase in length is given (to first order) by:

$$\delta L = \int_0^L \frac{1}{2} \left(\frac{\partial h}{\partial x} \right)^2 dx. \quad (\text{B.1})$$

We here define our Fourier transform as

$$h(x) = \frac{L}{2\pi} \int_{-\infty}^{\infty} dq (\tilde{h}_q) e^{+iqx}. \quad (\text{B.2})$$

By substituting Eq. (B.2) into Eq. (B.1), we get:

$$\begin{aligned} \delta L &= \frac{1}{2} \int_0^L \left[\int_{-\infty}^{\infty} dq (iq) h_q e^{iqx} \right]^2 \left(\frac{L}{2\pi} \right)^2 dx \\ &= \frac{1}{2} \int_{-\infty}^{\infty} dq 2\pi q^2 h_q^2 \left(\frac{L}{2\pi} \right)^2 \\ &\rightarrow \frac{1}{2} \left(\frac{2\pi}{L} \right) \sum_q 2\pi q^2 h_q^2 \left(\frac{L}{2\pi} \right)^2 \end{aligned} \quad (\text{B.3})$$

where $\tilde{h}_q$ was changed to h_q .

The increase in contour length results in the increase in the energy given by $\delta E = \gamma \delta L$, where γ is the line tension. Considering that all modes are independent and equipartition theorem is applied in thermal equilibrium, each q-mode has $k_B T/2$ of energy. Hence:

$$\frac{1}{2} k_B T = \frac{1}{2} \gamma q^2 \langle h_q^2 \rangle L \quad (\text{B.4})$$

which can be re-arranged to give:

$$\langle h_q^2 \rangle = \frac{k_B T}{L \gamma q^2} \quad (\text{B.5})$$

Therefore, what we have to do for measuring the line tension is (i) to detect the one-dimensional edge between the interface, (ii) to calculate the fluctuation amplitudes $\langle h_q^2 \rangle$ using Fourier transforms, and (iii) to fit the data by the theoretical equation Eq. (B.5).

Appendix C

Dynamic light scattering

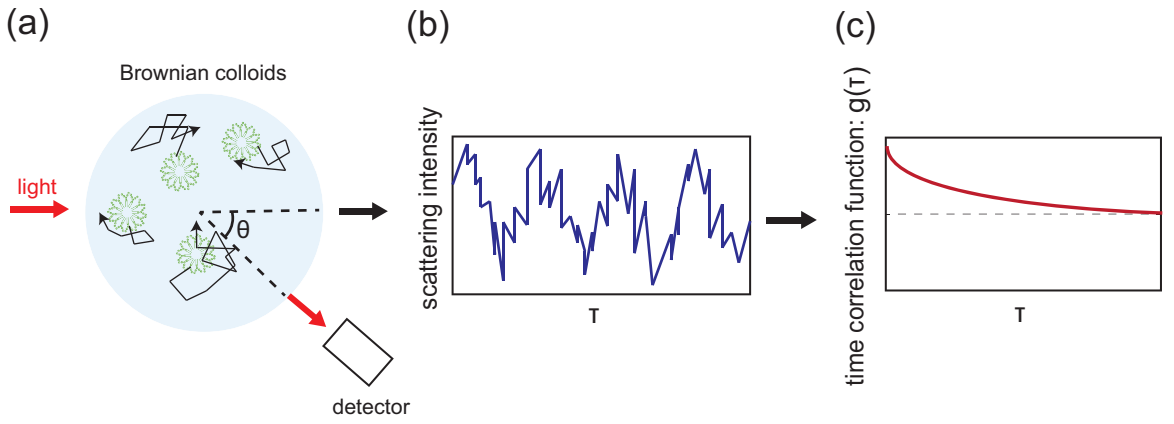


FIGURE C.1: Analysis steps of dynamic light scattering (DLS) experiments. (a) Schematic of DLS experiments. (b) Schematic of scattering intensity as a function of time. (c) Schematic of a typical time correlation function.

The second-order self correlation function $g(\tau)$ is obtained from the fluctuation of light scattered by colloids;

$$g(\tau) = 1 + a|G(\tau)|^2 \quad (\text{C.1})$$

where a and $G(\tau)$ are a constant and the first-order self-correlation function, respectively. $G(\tau)$ is written by

$$G(\tau) = \exp(-q^2 D t), \quad (\text{C.2})$$

where the parameter q and a translational diffusion coefficient D are

$$q = \frac{4\pi n_0}{\lambda} \sin \frac{\theta}{2}, \quad (\text{C.3})$$

and $D = \frac{k_B T}{6\pi\eta_0 R}$, respectively,

where n_0 is the refractive index of a solvent, e.g., water, λ is the laser wavelength, θ

is the scattering angle (see FIG. C.1), k_B is the Boltzman constant, T is the temperature, η_0 is the solvent viscosity, and R is the hydrodynamic radius. Therefore, the measured $g(\tau)$ can be theoretically fitted using by Eqs. (C.1)-(C.3), giving us the distribution of the hydrodynamic radii of the colloids.

Appendix D

Critical micelle concentration

Amphiphile molecules in a solvent dramatically self-assemble into micelle structures above a critical concentration. Consider an amphiphile solution in the dilute regime. The contribution of the molecules absorbed on the interface is now ignored. The chemical potential of micelles, composed of m amphiphile molecules, μ_m is given by:

$$\mu_m = \mu_m^0(T) + k_B T \ln x_m \quad (\text{D.1})$$

where x_m is the mole ratio of the micelles to the solvent. In the equilibrium state, the chemical potential per molecule is independent of the micelle size and constant. Thus, with regard to μ_m , the relation holds:

$$\mu_m^0(T) + k_B T \ln x_m = m(\mu_1^0(T) + k_B T \ln x_1), \quad (\text{D.2})$$

leading to

$$x_m = e^{-\frac{\mu_m^0 - m\mu_1^0}{k_B T}} (x_1)^m. \quad (\text{D.3})$$

The free energy per molecule, needed to make the micelle from m amphiphile molecules, g_m is given by:

$$g_m = \frac{\mu_m^0 - m\mu_1^0}{m}. \quad (\text{D.4})$$

The mole ratio of the amphiphile molecules x_0 in the solution is given by:

$$x_0 = \sum_m m x_m. \quad (\text{D.5})$$

Thus, using Eqs. (D.3) and (D.5) is rewritten by:

$$x_0 = \sum_m m e^{-\beta m g_m} (x_1)^m. \quad (\text{D.6})$$

From Eq. (D.6), we can obtain the relation between x_0 and x_1 . Above a critical concentration, x_1 becomes almost constant, implying that various sizes of micelles exist. In rough terms, note that the behavior does not depend micelle shapes, such as sphere, rod, and plate.

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