Journal of Colloid and Interface Science 343 (2010) 628–633

Contents lists available at ScienceDirect

Journal of Colloid and Interface Science

www.elsevier.com/locate/jcis

# Phase behavior in a ternary lipid membrane estimated using a nonlinear response surface method and Kohonen's self-organizing map

Yoshinori Onuki *, Kozo Takayama

*Department of Pharmaceutics, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan*

ARTICLE INFO

*Article history:*
Received 27 October 2009
Accepted 2 December 2009
Available online 6 December 2009

*Keywords:*
Lipid raft
Liposome
Fluorescence anisotropy
Response surface method
Kohonen's self-organizing map
Clustering

ABSTRACT

A novel method for investigating phase behavior in a ternary lipid membrane was developed and tested. Sixty-five model membranes composed of sphingomyelin (SM), dioleoyl phosphatidylcholine (DOPC), and cholesterol (Ch) were prepared, and fluorescence anisotropy between 25 °C and 60 °C was measured. Observed fluorescence anisotropy curves as functions of temperature were analyzed using a nonlinear response surface method and Kohonen's self-organizing map. Thus, we generated a scatter plot indicating the distribution of membranes with similar membrane properties. The scatter plot showed that the SM/DOPC/Ch membranes resolved into six clusters with distinct membrane properties. We then conducted differential scanning calorimeter (DSC) measurement of membranes typical of the clusters. The results indicated that the membranes consisted of several phase domains (i.e., $L_\alpha$, $L_\beta$, $l_o$ phase domains), and the clusters were distinguished by differences in the type and content of membrane domain. This method is accurate because the clusters were determined based on experimental values. This technique is useful for elucidating the phase behavior of ternary lipid membranes. These findings contribute to clarification of domain formation.



## 1. Introduction

A lipid raft is defined as a membrane microdomain rich in cholesterol (Ch) and sphingolipid located in the outer leaflet of the plasma membrane. Because numerous signaling molecules such as glycosylphosphatidylinositol (GPI)-anchored proteins [1,2] and receptor- [3,4] or nonreceptor-type tyrosine kinases [4–6] originate from these domains, the lipid raft is thought to act as a platform for protein segregation and signal transduction in the plasma membrane. Lipid-driven lateral separation of immiscible liquid phases is likely to be a crucial factor in the formation of lipid rafts in cell membranes [7]. Although the concept of a lipid raft has recently been widely accepted, its mode of formation in the cell membrane remains controversial.

Model membranes such as liposomes are effective tools for elucidating the formation of lipid rafts. Their lipid composition can be manipulated to suit the purpose of the experiment, and results obtained in this way are likely to be consistent. In addition, several membrane domains are known to coexist in model membranes as well as in cell membranes [8–12].

In general, lipid bilayers are classified into three different phases in order of increasing fluidity: a solid-ordered phase ($L_\beta$), a liquid-ordered phase ($l_o$), and a liquid-disordered phase ($L_\alpha$) [7,13]. The $L_\beta$ and $L_\alpha$ phases are also called gel and liquid crystalline phases, respectively. Ordered and tight packing are typical of the $L_\beta$ phase membrane, whereas fast axial rotation and high lateral mobility are observed in the $L_\alpha$ phase membrane. The Ch-rich membrane exists as an $l_o$ phase membrane; this phase is intermediate between the $L_\alpha$ and $L_\beta$ phases. Its ordered packing is similar to that of the $L_\beta$ phase, but its fast axial rotation and high lateral mobility are similar to that of the $L_\alpha$ state. The lipid raft is assumed to exist as an $l_o$ phase membrane in the plasma membrane.

A mixture of three different lipids, lipids with a high phase transition temperature ($T_m$) (e.g., lipid with saturated acyl chains), lipids with a low $T_m$ (e.g., lipid with unsaturated acyl chains), and Ch, is required to generate membrane domains. Such membranes have been widely used to mimic plasma membranes to elucidate the formation or structures of lipid rafts [9,11,12,14,15]. Even though these membranes are much simpler than biological mem-

*Abbreviations:* CFM, confocal fluorescence microscopy; Ch, cholesterol; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimeter; FRET, Förster resonance energy transfer; GPI, glycosylphosphatidylinositol; $L_\alpha$, liquid-disordered phase; $L_\beta$, solid-ordered phase; $l_o$, liquid-ordered phase; MVS, multivariate spline interpolation; RSM-S, response surface method incorporating multivariate spline interpolation; SM, sphingomyelin; SOM, Kohonen's self-organizing maps; $T_m$, phase transition temperature.

* Corresponding author. Fax: +81 3 5498 5783.
*E-mail address:* onuki@hoshi.ac.jp (Y. Onuki).

0021-9797/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.jcis.2009.12.009

branes, their phase behavior remains complicated. Despite numerous research studies, consensus on membrane phase behavior has yet to be reached. Veatch et al. generated a ternary phase diagram by observing the surface of giant unilamellar vesicles using confocal fluorescence microscopy (CFM) and described a condition in which phase separation occurs [11,12]. Although microscopic observation can be used to directly detect phase separation on membrane surfaces, this method has limitations. First, because giant unilamellar vesicles are not obtained from all lipid compositions, whole-phase behavior can never be determined with this method. Second, because the border region between membrane domains is thought to be an ambiguous structure, it is difficult to distinguish membrane domains by subjective evaluation. Third, because the phase behavior of ternary lipids is very complicated and is substantially changed by slight differences in lipid composition, a bunch of model membranes differing in lipid composition should be examined to elucidate phase behavior.

Fluorescence analyses such as Förster resonance energy transfer (FRET) and fluorescence anisotropy are also employed to identify the lipid phase behavior of membranes [14,16–19]. Because these measurements do not require preparation of a giant unilamellar vesicle, a wider range of lipid compositions can be examined than with CFM. In addition, measurements based on fluorescence analysis enable objective and quantitative evaluation of domain formation, and the experimental procedure is not very complicated. However, large data sets are required to fully understand the relationship between lipid composition and phase behavior and, in most cases, the collection of such large data sets is impractical.

To overcome this problem and elucidate the phase behavior of ternary lipids, we applied a response surface method incorporating multivariate spline interpolation (RSM-S) and a data mining technique (Kohonen's self-organizing maps; SOMs). Firstly, liposomes composed of sphingomyelin (SM), dioleoyl phosphatidylcholine (DOPC), and Ch were prepared and their fluorescence anisotropy was measured. Using data based on fluorescence anisotropy, we developed a scatter plot indicating the distribution of membranes with similar membrane properties. Response surface methods and a data mining technique were used to compensate for the lack of experimental data. Using these methods, we successfully resolved the membranes into several clusters. This is the first technical report on the phase behavior of lipids conducted using response surface and data mining methods.

## 2. Experimental procedure

### 2.1. Materials

Ch was purchased from Wako (Osaka, Japan). Chicken egg SM (Coatsome NM-10) and DOPC (Coatsome MC-8181) were purchased from Nippon Oil & Fat (Tokyo, Japan). More than 70% of the SM is 16:0 SM. 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were of analytical grade and are commercially available.

### 2.2. Preparation of liposomes

Liposomes composed of SM, DOPC, and Ch were prepared as reported previously [19]. In brief, designated amounts of lipids dissolved in chloroform were transferred to a flask, and the chloroform was removed by evaporation at room temperature under a stream of nitrogen. This procedure resulted in the formation of a thin lipid film on the wall of the flask. The film was stored overnight in a vacuum desiccator to ensure complete evaporation of the chloroform. Purified water (10 mL) was added to the flask, and the lipids were hydrated for 30 min. The total lipid concentration was adjusted to 10 mM. The suspension was sonicated for 10 min at about 60 °C using a bath-type sonicator. After the samples were cooled to room temperature, they were stored at room temperature for maximum of 2 days before use in the experiments.

### 2.3. Fluorescence anisotropy measurement

The liposome was labeled with DPH by adding 10 μL of 10 mM freshly prepared DPH stock solution in tetrahydrofuran to 1000 μL of liposome suspension and then incubating the mixture at 37 °C for 2 h in the dark to complete the labeling. The samples were diluted 50 times with purified water. The fluorescence anisotropy of DPH in the liposomes was measured using a fluorescence spectrophotometer (F-450; Hitachi, Tokyo, Japan) at an excitation wavelength of 351 nm and an emission wavelength of 430 nm. The temperature range during measurement was 25–60 °C. Steady-state fluorescent anisotropy was calculated using the following equation:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \tag{1}$$

where $r$ is anisotropy and $I_{VV}$ and $I_{VH}$ are the intensities measured parallel and perpendicular to the polarized exciting light, respectively. The $G$ factor was defined as $I_{VV}/I_{VH}$, which is equal to the ratio of the sensitivities of the detection system for vertically and horizontally polarized light. The $G$ factor of our detection system was 1.202.

### 2.4. Differential scanning calorimeter measurement

The liposome suspensions were lyophilized using a freeze dryer (FD-1; Tokyo Rikakikai, Tokyo, Japan) under reduced pressure. The freeze-dried liposomes (5 mg) were placed in aluminum pans for the DSC measurement. DSC measurements were performed using a Thermo Plus DSC 8230 instrument (Rigaku, Tokyo, Japan) equipped with a refrigerated circulator (Rigaku, Tokyo, Japan). The scan rate was set to 1 °C/min.

### 2.5. Clustering of SM/DOPC/Ch membranes into membranes with similar properties

The procedure for clustering membranes is shown in Fig. 1. Sixty-five model liposomes with different lipid compositions were prepared (Fig. 2). Fluorescence anisotropy was measured at temperatures ranging from 25 °C to 60 °C. dataNESIA software, version 3.0 (Yamatake Corp., Tokyo, Japan) was used for RSM-S. The observed fluorescence anisotropy values at 25, 30, 35, 40, 45, 50, 55, and 60 °C and the differences in values from 25 °C to 60 °C were used as tutorial data for generating response surfaces using RSM-S. Fluorescence anisotropy values of untested lipid compositions were predicted by reading points on the response surfaces. The number of untested lipid compositions for the prediction was 5041. Lipid composition and corresponding fluorescence anisotropy values for every 5 °C from 25 °C to 60 °C were regarded as an input data set. Namely, 5106 data sets, including experimental and predicted data, were used for SOM clustering. SOM clustering was performed using Viscovery software, SOMine version 4.0 (Eudaptics Software, Vienna, Austria). The number of nodes in the output was set at 2000. Thus, a scatter plot indicating the distribution of distinct membranes was developed using reference vectors for each cluster.