Membranomics Research on Interactions between Liposome Membranes with Membrane Chip Analysis

Toshinori Shimanouchi, Ena Oyama, Haruyuki Ishii, Hiroshi Umakoshi, and Ryoichi Kuboi*

Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University
1-3, Machikaneyama-cho, Toyonaka, Osaka 560-8531, Japan

The liposome immobilized on indium tin–oxide (ITO) electrode was prepared to evaluate the liposome–liposome interaction to develop “Membrane Chip”. Three kinds of standard liposomes entrapping the fluorescence probe, calcein, were used for their immobilization, where the immobilized liposomes show the different characteristics in their phase transition temperature and membrane fluidity. Membrane Chip could give the set of the quantitative information relating to the membrane–membrane interaction. The interaction between three immobilized liposomes and 22 kinds of liposomes as samples was then systematically investigated on a basis of the calcein release from the immobilized liposomes, which could give the information in relation to “Membranome”. It was, therefore, found that the Membrane Chip as a means of Membranomics research was effective to evaluate the Membranome information of liposomes.

Key words: membrane stress biotechnology / liposome–liposome interaction / multivariate analysis / membrane chip / liposome immobilization

1. Introduction

Recently, the approach to study the protein-lipid membrane interaction has been developed to clarify the membrane–related phenomena in cell system, such as the endocytosis, the exocytosis and the membrane fusion. On the other hand, the antioxidative activity or gene expression system have been well constructed on liposome membrane, which is called as LIPOzyme (liposome + enzyme). The previous reports on the LIPOzyme have indicated that the lipid composition and the phase state of lipid membrane, such as liquid–ordered (l_or) phase, liquid–disordered (l_d) phase and their mixture (l_d + l_or) phase, could control their function. Therefore, a systematic investigation on the membrane properties as a function of the lipid composition is expected to contribute to the understanding not only on the phenomena in cell system but also on the effective design of novel biomaterials such as LIPOzyme. The information as a whole in relation to the lipid composition, the membrane property, the phase state of lipid membrane, or the function induced on lipid (liposome) membrane could be called as “Membranome” according to the previous manner of genome or proteome. However, there is no report on the investigation, i.e. Membranomics research, to acquire the Membranome information.

In the Membranomics research, the adequate methodology is needed to evaluate the interaction of lipid (liposome) membrane with the biomaterias such as peptides/proteins or liposomes. In a series of reports, the immobilization technique of intact liposome on solid surfaces has been developed to apply to the investigation of the protein–liposome interaction, and the membrane-membrane interaction. In the study in relation to the protein–liposome interaction, the systematic information for the surface property of proteins could be acquired. Thus, a Membranome information of lipid (liposome) might be elucidated from a systematic investigation on membrane–membrane interaction. The approach with an arrayed–type chip immobilizing the liposomes as a
2. Materials and Methods

2.1 Materials

The phospholipids used were 1-palmitoyl–2-oleoyl–sn-glycero–3-phosphocholine (POPC), 1,2-dipalmitoyl–sn-glycero–3-phosphocholine (DPPC), 1,2-dimyristoyl–sn-glycero–3-phosphocholine (DMPC), 1,2-dioleoyl–sn-glycero–3-phosphocholine (DOPC), 1,2-distearoyl–sn-glycero–3-phosphocholine (DLCPO, saturated), 1,2-dilinoleoyl–sn-glycero–3-phosphocholine (D LPC(unsaturated)), Egg yolk phosphatidyl–ethanolamine (EPE), which were purchased from Avanti Polar Lipids (Birmingham, Wales, UK). Lissamine™ Rhodamine B 1,2-dihexadecanoyl–sn–glycero–3–phosphoethanolamine (Rh−DHPE) was purchased from Molecular Probe (Eugene, OR, USA). Indium tin–oxide (ITO) electrodes for the immobilization of the liposomes were purchased from BAS Co. Ltd. (Tokyo, Japan). sphingomyelin (SM), diacetyl glycerol (DAG), cholesterol (Ch), stearic acid (SA), linoleic acid (LA) and 16–Mercaptohexadecanoic acid was obtained from Sigma Aldrich (St.Louis, MO, USA). N-Hydroxysuccinimide (NHS) was purchased from Wako Pure Chemicals Co. Ltd. (Tokyo, Japan). The fluorescence probe, calcein and 1-ethyl–3-(3–dimethylaminopropyl)–carbodiimide hydrochloride (WSC) were purchased from Dojindo Laboratories (Kumamoto, Japan). The nonionic detergents Triton X–100 and cholesterol were purchased from Sigma (New York, NY, USA). All other chemicals of analytical grade were purchased from Wako Pure Chemical (Osaka, Japan).

2.2 Liposome preparation

As for the liposomes to be immobilized on the ITO electrode, three kinds of liposomes were prepared from POPC/EPE, DOPC/EPE, or DPPC/EPE, as previously described 9–11, 10. In brief, three kinds of lipid solutions, DPPC/EPE (99/1 mol%), DOPC/EPE (99/1 mol%), and POPC/EPE (99/1 mol%), were prepared in chloroform. They were dried in a 100 ml round–bottom flask by rotary evaporation under reduced pressure. The lipid film was dissolved in chloroform and the solvent was evaporated again to obtain a homogeneous lipid thin film. The obtained lipid film was kept under high vacuum for at least 3 hr and then hydrated with 100 μM calcein solution (pH 7.5) at room temperature to form multilamellar vesicles. After five time freezing–thawing treatment, the liposome size was adjusted by the extrusion of the suspension through polycarbonate membrane (pore size: 100 nm). Finally, the free calcein was removed by a gel permeation chromatography (Sepharose 4B, 11 cm × 1 cm). As for the immobilization experiment of the liposomes on the ITO electrode, POPC/EPE/Rh–DHPE (98/1/1 mol%) liposomes entrapping calcein were also prepared in a similar manner. As for the sample liposomes to be added on the liposome-immobilized ITO electrodes, 22 kinds of liposomes listed in Table 1, were prepared in Tris–HCl buffer (100 mM, NaCl 150mM, pH 7.5) in the similar preparation manner to the above, except for POPC/oxidized Ch (POPC/oxCh, 66/33 mol%) .

For the preparation of POPC/oxCh, POPC/Ch liposomes (66/33 mol%) were oxidized by mixing CuSO4/H2O2 solution over night. This liposome suspension was mixed with chloroform to extract both phospholipid and oxCh by Folch’s method 21). A lipid thin film of POPC/oxidized Ch was again formed and hydrated with the Tris–HCl buffer. The size of the liposomes was adjusted to 100 nm in diameter with the extrusion method after the above mentioned freezing–thawing treatment.
2.3 Immobilization of liposomes on an ITO electrode

The immobilized–liposome electrode was prepared according to the previous report \(^9\)–\(^{11,16}\). In short, a self-assembled monolayer (SAM) using 16-mercaptohexadecanoic acid was formed on indium tin-oxide (ITO) (1 cm × 1 cm). To activate the SAM membrane, the above electrode was immersed for 3~4 hours in a solution consisting of dioxane/distilled water (90/10, vol%) solution containing 17 mM N-hydrosyssuccinimide, 17 mM WSC. The liposome entrapping either the electrolyte or calcine then bound to the SAM layer by the amino conjugate method. After 1 hr, the ITO electrodes immobilizing three kinds of liposomes were rinsed with the Tris–HCl buffer.

2.4 Evaluation of liposome–liposome interactions

Liposome samples of 4 ml (10 μM lipid) were applied to the surface of the ITO electrode containing the above three kinds of immobilized liposomes. As an index of membrane–membrane interaction, the change of the fluorescence intensity of calcine on the electrode surface was evaluated by using an Olympus IX–51–11 FL/PH–S fluorescence microscope and an ORCA–ER C4742–95 CCD camera (Hamamatsu Photonics, Japan) connected to an Image analyzer.

3. Results and Discussion

3.1 Liposome Immobilization onto an ITO electrode for development of Membrane Chip

The liposomes were immobilized by amino coupling between the amino group of EPE in the liposome and the carboxyl group of thiol on the electrode (Fig. 1(a)). The immobilization of liposome with its high density could be achieved by the observation with an atomic force microscopy (Fig. 1(b)) and was estimated to be approximately 70–liposomes/μm². This immobiliza-
Calcein was entrapped inside liposome as a fluorescent probe in order to monitor the membrane–membrane interaction. After the immobilization of liposomes, the fluorescence derived from the entrapped calcein was observed on the ITO electrode (Fig. 1(c)). The addition of Triton X–100 made the fluorescence intensity disappeared on the electrode due to the disruption of the liposome structures by the detergent. The immobilization and stability of the liposomes were furthermore confirmed according to the previous method\(^9\). POPE/EPE/Rh–DHPE liposomes entrapping calcein were immobilized and the fluorescence of both Rhodamine and calcein were observed. The fluorescence of both molecules disappeared after the addition of Triton X–100 (data not shown). This indicates a complete disruption of liposomes and a removal of lipids from the electrode. Furthermore, the liposomes were added onto either bare ITO or onto a non-activated SAM membrane on ITO electrode. No fluorescence was observed in both cases (data not shown), suggesting that neither physical absorption of the liposomes nor amino coupling occurred without activation of the SAM.

From the above findings, it has been shown that the liposomes can be immobilized on the electrode with keeping the intact structure of liposome as shown in Fig. 1(a).
3.2 Analysis of the direct interaction between liposome membranes

The immobilized–liposome electrode has been reported to be useful for the study of the hydrophobic interaction between liposomes and proteins. It has been, furthermore, shown that the membrane–membrane interactions, including membrane fusion, were caused by the hydrophobic interaction between membranes, not only by the electrostatic interaction. These results were based on the study using the immobilized–liposome chromatography. It is, therefore, considered that the interaction between free liposome and immobilized liposome could be also evaluated by using the above liposome-immobilized electrode.

Fig. 1(a) shows the conceptual scheme of the direct interaction between an immobilized liposome and an added liposome (analyte liposome). The analyte liposome could perturb the lipid membrane of the immobilized liposomes, resulting in the release of the entrapped calcein from the immobilized liposomes. The influence of fluorescence intensity in the bulk suspension can be neglected because the released and diluted calcein had only little fluorescence outside liposomes as compared with that inside liposomes. A suspension of POPC SM/Ch (33/33/33 mol%) liposome was externally added as an analyte liposome to POPC–liposome immobilized on ITO electrode. As shown in Fig. 1(d), the calcein fluorescence intensity decreased after the addition of the analyte liposome, and reached a constant value within 10 min (Fig. 1(d)). In the case of the direct interaction between POPC liposomes and immobilized POPC liposomes, no decrease of fluorescence was observed. In the following, the observed change of calcein fluorescence (\(\Delta I\)) was used as a measure for the extent of liposome–liposome interaction. Since the initial fluorescence \(I_0\) depends on the number density of the immobilized liposome on the electrode surface, the membrane–membrane interaction was quantified by the relative fluorescence decrease, \(\Delta I/I_0\) (RF value). The RF value was defined as the \(\Delta I/I_0\) value.

Three kinds of immobilized–liposomes and 22 kinds of added liposome suspensions were used to systematically investigate the membrane–membrane interaction. The calcein release from the POPC, DOPC, and DPPC liposomes immobilized on the electrode was measured (RF\(_{POPC}\), RF\(_{DOPC}\), and RF\(_{DPPC}\)). Fig. 2(a) shows the RF spectra for calcein release induced by the immobilized liposome (RF\(_{POPC}\), RF\(_{DOPC}\), and RF\(_{DPPC}\) was measured).
re-plotted, showing the interaction profile for analyte liposomes (Fig. 2(b)). The RF-space should include the interaction model for the membrane–membrane interaction. Previously, the membrane property (membrane fluidity) has been reported to be roughly related to the passive diffusion of calcein across the liposome membranes 22) or to the membrane fusion 20). It is, therefore, considered that the interaction model hidden in the RF-space should be related to the membrane property. In the following, the possible interaction model for the membrane–membrane interaction was preliminarily investigated with a multivariate analysis.

3.3 Classification of liposomes based on the multivariate analysis

— Approach with a generalization of index for membrane–membrane interaction —

The cluster analysis prior to a classification of data set 23) was performed. The dendrogram for the data obtained is shown in Fig. 3(a) based on the Ward’s
method. According to the Milligan’s test with a Monte Carlo simulation, the classification of a data set with 22 samples into two or three groups is reasonable. Therefore, a dendrogram in Fig. 3(a) was classified into two main groups A and B, followed by the subgroups A1 and A2 in A.

Principle component analysis (PCA) is a useful method to understand the global characteristic of a profile of membrane–membrane interaction, as similarly shown in Fig. 2(b), because a PCA needs no pre-assumed interaction model to explain the phenomena. Fig. 3(b) shows the PC–plane obtained from Fig. 2. The result of a cluster analysis was superimposed on Fig. 3(b). The PC–plane could roughly be separated into 2 or 3-groups except for the liposome 18 which is expected to be assigned to group A2. This is because the calculation algorithm of Ward’s method could not avoid the assignment of the liposome 18 into group B, not A2.

In the theory of PCA, the principle component score could be described as

$$PC_1 = \sum_{j=1}^{n} \omega_{ij}RF_{ij}, \text{i.e.,}$$

$$PC_2 = \omega_{12}RF_{POP} + \omega_{13}RF_{DOPC} + \omega_{13}RF_{DPPC}$$

where \(\omega_{ij}\) is the weighted proportion with a condition of \(\omega_{11} + \omega_{12} + \omega_{13} = 1\) and \(j = 1, 2,\) and 3 mean POPC, DOPC, and DPPC liposomes, respectively.

The proportion \(\omega_{ij}\) can be determined by the eigen value problem of the dispersion matrix or the correlation matrix of the data set \(x_k \mid x_k = (RF_{k1}, RF_{k2}, RF_{k3}), k = 1 \sim 22\). The PC1 and PC2 values are thought to be the generalized parameters for RF values used here because PC1 (i = 1, 2) are connected with the RF value in eqs.(1) and (2). From the PCA, \(\omega_{11} > 0, \omega_{12} > 0,\) and \(\omega_{13} < 0\) were obtained, indicating that PC1 might imply the calcein release dependant of the phase state of membrane surface. POPC and DOPC showed \(l_0\) phase and DPPC showed \(s_0\) phase (Table 1). The permeation behavior under \(l_0\) or \(l_0\) phase condition depended on the membrane fluidity. The anomaly strong permeation was, however, observed in the liposome with the mixture \(l_0 + l_0\) phase. Thus, it is considered that PC1 is the index for the surface state of liposome membrane enough to induce the characteristic interaction with the immobilized liposomes.

The construction of a robust library is the important issue at the development of screening using the array systems. The huge library guaranteed the interpretation of screening experiments such as the gene library of \(10^6\sim10^9\) clones or the peptide library of 100–200 (4–8–mer peptides). The analysis of coefficient of variation (CV) or correlation coefficient is reliable for the assessment on robustness of the aforesaid huge library. However, the liposome membranes have the difficulty in determination of library size in terms of lipid compositions and the molar ratio of mixture. Therefore, a robustness analysis of liposomes library with not a large size was directly performed by the fluctuation of the coordination in PC–plane like Fig. 3(b). Accordingly, the fluctuation of the coordination \(y_k(PC1, PC2)\) (\(k = 1\sim22\)) by adding new data into the ordinal data set was can be simply estimated by the following parameter V:

$$V = \Sigma_{k=1}^{n} \mid y_k' - y_k \mid^2/n$$

where \(n\) is the sample number of data set, \(y_k'\) is the point after transfer of \(k\)–th point \(y_k\) in PC–plane by an addition of \(y_{k+1}\). The smaller the V value is, the more stable the data set becomes, which is corresponding with the property of conventional CV. Thus, the V value is possibly reasonable for the assessment on the robustness of the data set. The index for the robustness of data set, V, was plotted against the size of data set, \(n\) (Fig. 3(c)). The V value decreased with increasing the size of data set nevertheless to the species of liposome as an additive, implying that the extension of data set guarantees its robustness.
3.4 Possible interpretation of the principle components

In a PCA method, the condensed information on the focusing data set could be elucidated from the PC–plane. Since the accumulated proportion of PC1 was over 50%, the global characteristic of a data set \( y_k \) could roughly be discussed by using the PC1 value. The PC2 value with low proportion (20%) was, however, ruled out from the discussion. In order to evaluate the common feature for liposomes at the membrane-membrane interaction, the PC1 values for 22 kinds of liposomes were plotted in its order (Fig. 4). Here, the PC1 value means the calcein release by distinguishing the phase state of liposomes. The ratio of liposome including cholesterol in group B (83.3%) was significantly larger than those of group A (25.0%). Also, 83.3% of liposomes were the binary or ternary lipid system in group B whereas 62.5% of liposomes were single lipid system in group A. This indicates that the liposome with cholesterol–rich domain and the lipid with unsaturated fatty acids favored to interact with the liposome with \( l_d \) phase. On the other hand, the liposomes in group A2 preferably interacted with the DPPC liposome with non–\( l_d \) phase (presumably solid–ordered (s\(_d\)) phase). Therefore, the liposome with cholesterol-rich domain might possess the ability to recognize the phase state such as \( l_d \), \( l_o \), or \( s_o \) phase, which is the first finding to our knowledge. It was confirmed that the above feature was robust in the data set with less than 20 kinds of liposomes because of the robustness of the data set of \( y_k \) (data not shown).

Furthermore, it can be considered that PC1 can be utilized as an index for the cholesterol–rich domain advantageous for the interaction between membranes. Recent study has reported that Niemann–Pick type C disease and Alzheimer’s disease are closely related to the elevation of cholesterol inclusion within neuronal cell membranes \(^{32}\). The model cell membrane (No.22 in Table 1) showed smaller PC1 value than the liposomes with cholesterol in domains. Thus, Membrane Chip analysis is expected to assess the abnormality of the biomembranes (due to the increase of cholesterol) although the further research in detailed are needed.

4. Conclusion

The experimental results obtained in this work suggest that the liposome immobilized on the ITO electrode can be utilized as a useful tool for the detection of differences in the surface states of liposome. The liposomes used here could be divided into two or three groups in terms of the membrane-membrane interaction. Membrane library with 22 kinds of liposomes was robust against the statistical treatment and could clarify the global characteristic that the liposome with cholesterol–rich domain could preferably interact with the liposome with \( l_d \) phase. This feature is considered to give the global characteristics of the RF–space. Therefore, a significance of the “Membranome” information and its acquirement with Membrane Chip (Membranomics research) could be demonstrated although the further investigation of preparation of Membrane Chip and the size of a data set are needed for the high through–put treatment. The global characteristic of the membrane library clarified in this study is expected to be applicable to the field of the liposome–based bioproduction system (e.g. LIPOzyme) or the selection of optimal liposome as a drug delivery system.

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