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Relationship between the reconstituted vesicle size and the transmembrane protein-to-lipid ratio

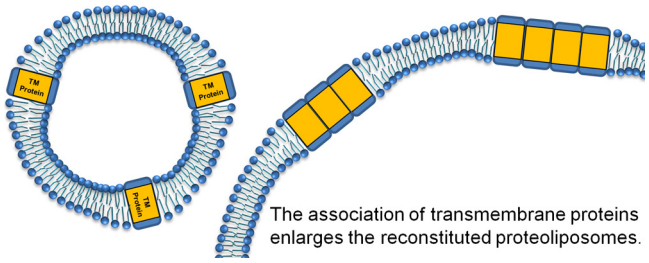
Noritaka Kato[a,b,*], Shunsuke Kimura[a], Takanori Sasaki[a,c]

[a] Graduate School of Science and Engineering, Meiji University, Kawasaki 214-8571, Japan
[b] School of Science and Engineering, Meiji University, Kawasaki 214-8571, Japan
[c] School of Interdisciplinary Mathematical Sciences, Meiji University, Tokyo 164-8525, Japan

HIGHLIGHTS

- The TM proteins prevented the formation of the lipid aggregates.
- The vesicle size changed discreetly at a certain ratio of lipid to TM protein.
- Thermodynamic considerations were made to explain the above phenomena.

GRAPHICAL ABSTRACT



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ABSTRACT

The lipid vesicles have been reconstituted with the transmembrane proteins, which are the trimer of the seven-transmembrane (7TM) proteins, by extracting the detergents from the protein/lipid/detergent mixture using detergent-adsorbent resin beads. It has been found that the size of the reconstituted vesicles significantly depends on the molar ratio of lipid to the protein (R_L). 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) or 1-palmitoyl-2-oleoyl-sn- glycero-3-phosphocholine (POPC) were used for the reconstitution. The size distribution of the reconstituted vesicles based on DMPC was narrowed by the insertion of 7TM proteins, suggesting that the protein insertion promotes the formation of unilamellar lipid bilayer rather than multilayered assemblies or undefined aggregates. In the case of POPC, the diameter of the reconstituted vesicles depended discreetly on the R_L. Upon increase in the R_L from 700 to 800, the average diameter jumped *ca.* three times larger. By assuming that this enlargement is driven by a phase separation between POPC and the 7TN proteins, the interaction energy between the inserted protein and the POPC bilayer was estimated.

1. Introduction

One third of proteins encoded in a human genome are membrane proteins [1], and more than half of pharmaceutical targets correspond to the membrane proteins [2]. In natural cells, membrane proteins are embedded in the fluidic lipid bilayer that is heterogeneous and dynamic. Such complicated situation prevents the direct investigation of the membrane proteins. To understand their chemical and biological functions, not only the extraction and purification of the membrane proteins from the cell but also the reconstitution of them into the model lipid bilayer are required [3]. Because the membrane proteins can only be solubilized in an aqueous phase by detergents due to their amphiphilic nature, a precise removal of the detergent from the mixture of detergent/protein/lipid, where all the amphiphyls were solubilized in an aqueous phase, is a common strategy to reconstitute the

* Corresponding author at: Tel./fax: +81 44 934 7292.
E-mail address: nkato@isc.meiji.ac.jp (N. Kato).

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membrane protein-containing lipid vesicles (proteoliposome). Along tremendous efforts for the extraction and purification, numbers of membrane proteins have also been reconstituted into the lipid bilayers [3], and it was found that the conformation and dynamics of the proteins in the membrane are fundamentally important, since the difference in these properties can alter their functions. In the case of transmembrane (TM) proteins, it had been pointed out that the degree of hydrophobic mismatch (the difference between the hydrophobic thickness of a lipid bilayer and that of TM protein) plays an important role in the proteoliposomes [4–6]. The mismatch induces an elastic stress to the lipid bilayer and/or the inserted TM proteins, resulting in the changes in lipid dynamics [7] and protein conformation [8], functions [9] and association [10].

Besides the analyses of the physico-chemical and biological functions of the individual proteins or lipids in the proteoliposomes, one could anticipate that the property of a biomembrane as a collective of lipids and proteins is also modulated by the method and condition of the reconstitution. Thus, we zoomed out from molecular to macroscopic level and focused on the diameter of the reconstituted vesicles, i.e., the macroscopic morphology. The dependences of the vesicle diameter on the lipid species and the ratio of lipid to TM protein (R_L) have been systematically investigated. 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) or 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were used for the lipid component. Halorhodopsin from *Natronomonas pharaonis* (NpHR) was used as the TM protein component. NpHR consists of the three seven-transmembrane proteins similar to bacteriorhodopsin and acts as a light-driven anion pump [11]. The trimeric structure of the NpHR is stable even in the detergent-containing solution as long as the buffer contains NaCl [12]. The reconstitution of the lipid/protein vesicles was performed by the extraction of the detergents from the protein/lipid/detergent mixture (the solubilized system) using detergent-adsorbent resins, which is one of the popular reconstitution methods [3]. It was found that the diameter of the reconstituted vesicles significantly depended on the R_L and the dependence in the DMPC based vesicles was different from that in the POPC ones. The influence of the TM protein (NpHR) upon the reconstitution of proteoliposomes and the protein–lipid interaction were discussed based on the hydrophobic mismatch.

2. Experimental details

NpHR with C-terminal histidine tag was expressed in *Escherichia coli* BL21-(DE3) cells transformed by pET-21c vector [13]. The membranes of the grown cells were solubilized by 30 mM *n*-dodesyl-β-D-maltoside (DM). NpHR was purified by affinity chromatography on a nickel–nitrilotriacetic acid (Ni–NTA) agarose column (Qiagen GmbH), and NpHR (20 to 10 μM) in 0.1% MOPS buffer containing 150 mM NaCl and 5% DM (**Buffer-1**) was obtained. The medium and the detergent were then exchanged by Tris–HCl buffer (pH 7.5, 5 mM) containing 150 mM NaCl and 4.5 mM TritonX-100 (**Buffer-2**). The NpHR-containing **Buffer-1** was loaded to the PD-10 column packed with Sephadex G-25 (GE Healthcare Life Sciences) up to 2.5 mL and NpHR was eluted by **Buffer-2**, and the collected NpHR solution was loaded and eluted again using the PD-10 column to ensure the complete exchange of buffer. Specifically, the fluorescent detergent, TritonX-100, was used to monitor the removal of detergent during the reconstitution. The concentration of the NpHR in **Buffer-2** was determined by using the extinction coefficient of 54,000 M^{-1} cm^{-1} at 578 nm [12].

Before the preparation of the TritonX-100/NpHR/lipid mixture, the vesicles were made by the lipid solely. The chloroform solution of lipid (DMPC or POPC, NOF Corporation, Inc., Japan) was dried and a lipid film was formed inside the vial, followed by vacuum evaporation for at least 12 h. The Tris–HCl buffer (pH 7.5, 5 mM) containing 150 mM NaCl (no TritonX-100, **Buffer-3**) was added and sonicated to obtain a suspension. This suspension was then pushed through a polycarbonate film (100 nm pore size) 12 times using an Avanti mini-extruder at room temperature to obtain the lipid vesicles with 100 nm diameter. The equal amounts of the vesicle-containing **Buffer-3** and the NpHR-containing **Buffer-2** at the adjusted concentrations of lipid and NpHR were added, and finally 2 mL of the TritonX-100 (2.25 mM), NpHR and lipid mixture in the Tris–HCl buffer (pH 7.5, 5 mM) containing 150 mM NaCl was prepared. The concentrations of DMPC and POPC in the TritonX-100/NpHR/lipid mixture were ranged from 0.3 to 1 mM and 0.5 to 1.5 mM, respectively. The solubilization of the vesicles at these lipid concentrations using 2.25 mM TritonX-100 was confirmed by observing the disappearance of the particle size distribution at the average diameter of 100 nm using dynamic light scattering (DLS).

To remove TritonX-100, 40 mg of Bio-Beads (MS-2, Bio-Rad Laboratories, Inc.) as the detergent-adsorbent resins was added to the mixture every an hour for 4 times, resulting in the weight ratio of Bio-Beads (160 mg in total) to TritonX-100 in the mixture ≫40. During the removal of TritonX-100, the 2-mL tube containing the mixture and Bio-Beads was gently rotated less than 60 rpm.

One hour later after each addition of Bio-Beads, the fluorescence spectrum of TritonX-100 in the mixture was observed by the spectrofluorimeter (FP-6500, Jasco Corp.). TritonX-100 was excited at 270 nm, and the fluorescence spectra from 290 to 500 nm were recorded at a scan speed of 200 nm/min and a step resolution of 0.1 nm with blocking unwanted signals using a long pass filter (300 ± 5 nm). Because of this filter, the spectral shape at the shorter wavelength (<305 nm) was deformed.

After the removal of the detergents, the size distribution of the reconstituted vesicles was observed by the DLS method using the particle size analyzer (ELSZ, Otsuka Electronics Co., Ltd.). The light source was a semiconductor laser (660 nm) and the 165° backscattered light was observed. The self-correlation function was accumulated 70 times, and the distribution of scattered intensity versus particle diameter was obtained by the Marquardt method [14]. The minimum sample volume required for the observation was 0.5 mL.

To confirm the insertion of NpHR to the lipid membrane, the gel filtration was carried out using PD-10 column. After loading the 2-mL dispersion and being settled, 0.5 mL of **Buffer-3** was added and the eluted fraction (0.5 mL) was collected, and this addition-and-collection sequence was repeated. The eluted amount of the vesicles in each 0.5-mL fraction was evaluated by measuring the scattered light intensity using the particle size analyzer. The elution of NpHR was monitored by the observation of the NpHR absorbance. The visible extinction spectra of the dispersions were observed using the fiber optic UV–visible spectrometer (EPP2000C-50um, StellarNet, Inc.) with the UV–visible S2D2 fiber light source (L10671, Hamamatsu Photonics K.K.). The peak absorbance of NpHR located at 574 nm was obtained by subtracting the scattering component from the extinction spectrum. A cuvette with optical path of 10 mm and width of 3 mm with black walls (Black Micro Cell) was used, and the minimum sample volume required for the observation was 1 mL. Therefore the eluted amount of NpHR in each 1-mL fraction was evaluated, instead of 0.5 mL.

3. Results and discussion

The hydrocarbon thicknesses of the bilayers formed by DMPC and POPC are 25.4 Å and 27.1 Å, respectively [15]. The hydrophobic length of NpHR would be the same as that of bacteriorhodopsin (30–31 Å) [4,7], because their molecular structures are very similar

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