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Membrane fusion between baculovirus budded virus-enveloped particles and giant liposomes generated using a droplet-transfer method for the incorporation of recombinant membrane proteins

Misako Nishigami [a], Takaaki Mori [a], Masahiro Tomita [a], Kingo Takiguchi [b], Kanta Tsumoto [a,*]

[a] Division of Chemistry for Materials, Graduate School of Engineering, Mie University, 1577 Kurimamachiya-cho, Tsu, Mie 514-8507, Japan
[b] Division of Biological Science, Graduate School of Science, Nagoya University Furo-cho, Chikusa, Nagoya 464-8602, Japan

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ABSTRACT

Giant proteoliposomes are generally useful as artificial cell membranes in biochemical and biophysical studies, and various procedures for their preparation have been reported. We present here a novel preparation technique that involves the combination of i) cell-sized lipid vesicles (giant unilamellar vesicles, GUVs) that are generated using the droplet-transfer method, where lipid monolayer-coated water-in-oil microemulsion droplets interact with oil/water interfaces to form enclosed bilayer vesicles, and ii) budded viruses (BVs) of baculovirus (*Autographa californica* nucleopolyhedrovirus) that express recombinant transmembrane proteins on their envelopes. GP64, a fusogenic glycoprotein on viral envelopes, is activated by weak acids and is thought to cause membrane fusion with liposomes. Using confocal laser scanning microscopy (CLSM), we observed that the single giant liposomes fused with octadecyl rhodamine B chloride (R18)-labeled wild-type BV envelopes with moderate leakage of entrapped soluble compounds (calcein), and the fusion profile depended on the pH of the exterior solution: membrane fusion occurred at pH ~4–5. We further demonstrated that recombinant transmembrane proteins, a red fluorescent protein (RFP)-tagged GPCR (corticotropin-releasing hormone receptor 1, CRHR1) and envelope protein GP64 could be partly incorporated into membranes of the individual giant liposomes with a reduction of the pH value, though there were also some immobile fluorescent spots observed on their circumferences. This combination may be useful for preparing giant proteoliposomes containing the desired membranes and inner phases.

1. Introduction

In living cell systems, membrane fusion plays key roles in several phenomena, including endocytosis, exocytosis, fertilization, cell division, viral infection, etc. These phenomena have very frequently been investigated from biochemical, biophysical and physicochemical perspectives using artificial membrane vesicles (liposomes) [1,2]. Especially, with regard to endocytosis and exocytosis, it is very clear that membrane fusion in an intracellular system is necessary for the transport of membrane proteins without changes in their topological orientations. Cell-sized giant liposomes (GLs) or giant vesicles (GVs; in most cases, giant unilamellar vesicles (GUVs)) [3] have often been used to study the functions of membrane proteins, since they are so large (cell-sized, ~10–100 μm) that artificial membranes where the proteins are reconstituted can be directly observed using optical/fluorescence microscopy [4,5]. Several procedures for preparing giant proteoliposomes have been described [4]; these usually involve the incorporation of membrane proteins into lipid bilayers through membrane fusion [6–14]. In some cases, giant liposomes can be changed into giant proteoliposomes through membrane fusion with smaller, conventionally generated proteoliposomes with diameters of ~100 nm; for instance, membrane fusion is caused by the conjugation of fusogenic peptides to smaller liposomes [6] or highly concentrated proteoliposomes that coalesce under centrifugation [7]. In other cases, such proteoliposomes can be made to fuse with each other under a dry condition and are then rehydrated and grown to be cell-sized under an alternating electric field [8]. Membrane fusion due to the use of dehydration and rehydration [9,10], which has been widely used since the onset of such research, osmotic shock [11], freezing and thawing [12], and the use of gel-assisted composites [13,14], can also result in the formation of giant proteoliposomes.

* Corresponding author.
E-mail address: tsumoto@chem.mie-u.ac.jp (K. Tsumoto).

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Transmembrane proteins that have been expressed with cell-free recombinant expression systems can also be inserted directly into liposome membranes [15,16].

In previous studies, we reported an alternative procedure for the preparation of conventional (large unilamellar vesicles, LUVs) [17–19] and giant proteoliposomes [19–23] based on a recombinant gene expression system with budded viruses (BVs) of baculovirus and insect cells (Sf9 cells). Since baculovirus BVs propagate by repeated infection through endocytosis in host cells, they endogenously express the fusogenic glycoprotein GP64 on their envelopes, which is activated upon a reduction of pH in endosomes to induce membrane fusion between their envelopes and endosomal membranes [24]. In our first report, we used the gentle hydration (natural swelling) method [3] to prepare giant liposomes, and demonstrated fusion between fluorescent-labeled wild-type BVs and single giant liposomes as well as the dependence of this fusion on the pH and an acidic lipid composition, as in a natural setting [20]. Using this procedure, we successfully incorporated desired recombinant transmembrane proteins such as adrenergic receptor β [20] and thyroid-stimulating hormone receptor [19] (members of the family of G protein-coupled receptors (GPCRs); seven-span transmembrane protein receptor with signal transduction pathways), connexin 43 proteins (four-span transmembrane protein components to form gap junctions) [21], N-cadherin (single-span transmembrane protein of cell adhesion molecule (CAM) proteins) [22], and adenylate cyclase 6 (AC6) (12-span transmembrane protein effector with GPCR pathways) [23], each of which was expressed on envelopes of recombinant BV particles, into bilayers of giant liposomes through membrane fusion.

The gentle hydration method we adopted is so simple that it is still often used by many researchers. On the other hand, if one intends to encapsulate soluble materials within the inner aqueous phases of giant liposomes with a high entrapment efficiency, the droplet-transfer method (which uses stabilized W/O microemulsion droplets) [3,16,25–27] is more suitable. This method can produce giant liposomes in which both the lipid compositions for each leaf of the bilayers and the concentrations of materials in the interior are well-controlled, and thus it has potential for producing micro-vessels for use in artificial cell systems.

In the present study, we revisited membrane fusion that occurs when baculovirus BVs interact with giant liposomes that are prepared using the droplet-transfer method in place of gentle hydration. The fusogenic glycoprotein GP64 expressed on BVs can be activated when the pH of its surroundings is changed from neutral to acidic. With a confocal laser scanning microscope, we observed, for the first time, single giant liposomes fused with fluorescent dye (octadecyl rhodamine B chloride (R18))-labeled BVs with the moderate leakage of entrapped soluble compounds (calcein) and then obtained a profile of membrane fusion that depended on pH. After verifying these results, we examined the incorporation of recombinant transmembrane proteins, both a red fluorescent protein (RFP)-tagged viral envelope protein (GP64) and an RFP-tagged GPCR (corticotropin-releasing hormone receptor 1, CRHR1) into membranes of the giant liposomes. We found that these proteins were unevenly distributed on the droplet-transferred giant liposomes, and we did not observe such uneven patterns on giant liposomes prepared using the gentle hydration in our previous study [23].

2. Materials and methods

2.1. Materials

1,2-Dioleoly-*sn*-glycero-3-phoshocholine (dioleoylphosphatidylcholine, DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol, sodium salt (dioleoylphosphatidylglycerol, DOPG) for the preparation of liposomes was purchased from NOF Corporation (Tokyo, Japan) and dissolved in chloroform to prepare stock solutions. Calcein and octadecyl rhodamine B chloride (R18) were purchased from Life Technologies (Carlsbad, CA) to stain the inner aqueous phases of liposomes and the envelopes of BVs, respectively. Mineral oil (Sigma-Aldrich, St. Louis, MO) was used to prepare micro water-in-oil (W/O) droplets to form giant liposomes. For buffer solutions, Tris (2-amino-2-hydroxymethyl-1,3-propanediol), hydrochloric acid (HCl), acetic acid (CH_3COOH) and sodium acetate (CH_3COONa) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Other reagents were obtained commercially and of analytical grade.

2.2. Preparation of baculovirus

Budded viruses (BVs) of baculovirus (*Autographa californica* nucleopolyhedrovirus (AcNPV)) are viral particles with envelopes that are collected from the supernatant of a liquid culture of infected Sf9 cells [17,28]. BVs are around ∼100 nm in size and assume a matchstick-like shape [28–30]. Their gene components and lipid compositions have also been known [29,30]. The procedure used to prepare BVs is described below. All procedures were conducted at room temperature except as specifically mentioned.

Sf9 cells were first cultured with 5 mL of SF900 III serum-free medium (Life Technologies) in two 25-cm^2 plastic flasks for 3–4 days to obtain confluent cells. Cells were gently scraped and pipetted, and the 10-mL cell culture was put into a disposable baffled bottle (Corning, Corning, NY) with 100 mL of fresh SF900 III medium and cultured at 27 °C for 6–8 days in an incubator equipped with a shaker (150 rpm; Taitec, Koshigaya, Japan). The wild-type or recombinant AcNPV BV suspension was added to the bottle at around one MOI (multiplicity of infection) and cultured for an additional 4–5 days. The cells and debris were removed from the infected culture medium by centrifugation and the supernatant was ultracentrifuged at ∼107,000 × *g* (with a maximum radius of 152.5 mm, 25,000 rpm, 30 min, 15 °C; Beckman L-70 with an SW32Ti rotor, Beckman Coulter, Brea, CA) to form a pellet of BVs. The BVs were resuspended with modified PBS buffer (1 mM Na_2HPO_4, 10.5 mM KH_2PO_4, 140 mM NaCl, 40 mM KCl; adjusted to pH 6.2) and the suspension was placed on a step-wise sucrose gradient (10 w/w% (6.3 mL), 15 w/w% (6.3 mL) and 20 w/w% (18.9 mL) in PBS (pH 6.2) in a Beckman Ultra-Clear centrifuge tube) and then subjected to centrifugation (∼28,000 × *g* (with an average radius of 109.7 mm, 15,000 rpm), 30 min, 15 °C; Beckman L-70 with an SW32Ti). BV particles accumulated in two zones that consisted of solutions that contained 10% and 15% sucrose, respectively. The upper fraction (with 10% sucrose) contained damaged BV envelopes without viral DNAs and the lower fraction (with 15% sucrose) contained intact BV-enveloped particles with viral DNAs [17,28]. BV suspensions were recovered from both zones separately using syringes. Each fraction was diluted with PBS (pH 6.2) and ultracentrifuged again (25,000 rpm, 30 min, 15 °C; Beckman L-70 with an SW32Ti) to form a viral pellet. Finally, the pellets were resuspended with ∼150–300 μL of PBS (pH 7.5 (adjusted in advance with NaOH) for a wild-type case and pH 6.2 for a recombinant case). Hereafter, we refer to BVs collected from the upper and lower fractions as "upper-fraction BVs" and "lower-fraction BVs", respectively.

BVs labeled with R18 were prepared by the addition of an R18/ethanol solution [17]. Briefly, an aliquot of the R18 solution (4 mM in ethanol) was added to the BV suspension at a ratio of 40 nmol of R18 per 1 mg of protein of BVs. The mixture was then vortexed briefly and mixed with inversion for an hour. The protein concentration of the BV suspension was determined using a Protein Assay Kit (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as a standard. Samples of R18-labeled BVs were loaded onto a col-