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A method of gentle hydration to prepare oil-free giant unilamellar vesicles that can confine enzymatic reactions

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ABSTRACT

We report a new and improved method to prepare, by gentle hydration of lipid films, oil-free giant unilamellar vesicles (GUVs), in which enzymatic reactions can be encapsulated. The traditional method of gentle hydration requires very low concentrations of metal ions, whereas enzymatic reactions generally require mono- and divalent metal ions at physiological concentrations. In order to improve the production of oil-free GUVs that can confine enzymatic reactions, we developed a novel method also based on gentle hydration, but in which the precursor lipid film was doped with both 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEGylated lipid) and sugar. Close examination of the size, shape, and lamellarity of vesicles prepared in this manner demonstrated that the process improves the production of oil-free GUVs even at low temperatures and physiological salt concentrations. PEGylated lipid and sugar were found to synergistically improve GUV formation. Finally, we demonstrate the successful enzymatic synthesis of RNA within oil-free GUVs that were prepared on ice.

1. Introduction

A giant unilamellar vesicle (GUV) is a single, closed lipid bilayer membrane of diameter >1 μm. GUVs have been used to study biomembranes, membrane proteins, and models of living cells, and utilized in synthetic biology [1–11]. In these studies, GUVs are often used as systems to mimic cells.

The water in oil (W/O) emulsion centrifuge method [12] and the oil-supported microfluidic method [13–15] are the standard methods to prepare GUVs that can confine enzymatic reactions. These techniques can generate GUVs in the presence of metal ions at concentrations required for enzymatic reactions. The encapsulation yields of these W/O droplet-based methods are generally high. The lipid-coated ice droplet hydration method recently developed, for instance, obtains very high entrapment yields for water-soluble enzymes into giant vesicles (GVs) [16]. However, as large amounts of oil are used, it could potentially penetrate GUV membranes. In fact, the microfluidic jetting technique, an oil-supported microfluidic method, produces GUVs contaminated with oil [13,17]. Oil contamination may affect membrane properties such as thickness, stability, and permeability, as well as the activity of integral membrane proteins. Therefore, an oil-free method to prepare GUVs is urgently needed to investigate confined enzymatic reactions and active proteins.

Gentle hydration of lipid films is a traditional technique to prepare oil-free GUVs [18]. This technique gives a lower encapsulation yield than the W/O droplet-based schemes, but the GUVs prepared do not contain any oil at all. However, the technique is not compatible with enzymatic reactions, as it requires very low concentrations of metal ions (<0.1 mM) [19], whereas enzymatic reactions generally require physiological concentrations. Electroformation improves this technique by enabling GUV preparation at physiological concentrations of metal ions [20,21]. However, there is no report of GUVs prepared with electroformation at a low temperature, such as on ice, and at physiological concentrations of mono- and divalent metal ions. GUV preparation on ice is often required to encapsulate enzymatic reactions and active proteins. The agarose matrix-assisted procedure [22], which is another form of gentle hydration, also enables GUV preparation at physiological concentrations of mono- and divalents. However, GUV preparation through this technique has also not been tested on ice; furthermore, GUVs are typically attached to a surface, and are often arrayed in several layers above the surface [22]. These features would not be suitable for some applications.

Abbreviations: PSGH, PEGylated-lipid-and-sugar-doped gentle hydration; GUV, giant unilamellar vesicle; GMV, giant multilamellar vesicle; PEGylated lipid, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]; Nile red, 9-(diethylamino)-5 H-benzo(α)phenoxazin-5-one

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Thus, current methods of gentle hydration should be further improved.

Here, we describe a novel process of gentle hydration, both at physiological levels of mono- and divalent ions and low temperatures. The method is termed PEGylated lipid-and-sugar-doped Gentle Hydration (PSGH) of lipid films; the defining characteristic of the technique is the doping of lipid films with PEGylated lipid and sugar. GVs prepared using this method were closely examined by microscopy and flow cytometry to characterize size, shape, and lamellarity. Consequently, we found that PSGH improved the production of oil-free GUVs even at physiological concentrations of metal ions and low temperatures. A synergistic effect of PEGylated lipid and sugar on GUV productivity was clearly demonstrated. We also demonstrated the successful enzymatic synthesis of RNA within GUVs prepared on ice.

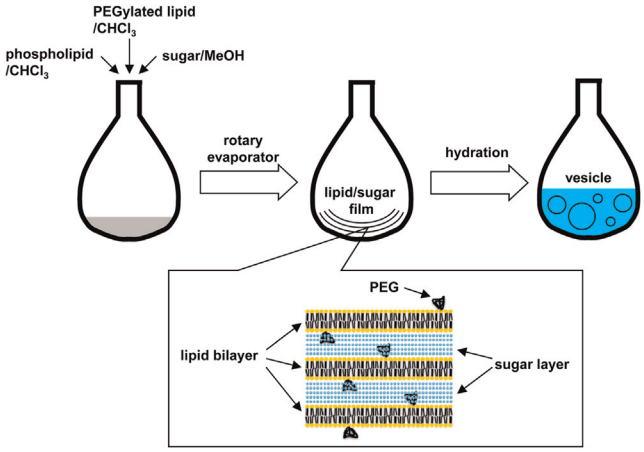

Fig. 1. Outline of PEGylated lipid-and-sugar-doped gentle hydration (PSGH).

2. Materials and methods

2.1. Reagents

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DOPE-PEG_{2000}: PEGylated lipid) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). D-Fructose and 9-(diethylamino)-5H-benzo(α)phenoxazin-5-one (Nile red), a red fluorescent dye, were obtained from Wako Pure Chemical Industries (Osaka, Japan). Solutions of 1 M Tris–$HCl_{(aq.)}$ (pH 8.0), 500 mM $MgCl_{2\ (aq.)}$, and 500 mM ethylenediamine-*N,N,N,N*-tetraacetic acid ($EDTA_{(aq.)}$ pH 8.0) were from Nacalai Tesque (Kyoto, Japan). Mag-Fluo-4 was procured from Invitrogen (Carlsbad, CA, U.S.A.). An NTP mix was purchased from New England Biolabs (Ipswich, MA, USA), while bovine serum albumin (BSA) and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Thermo T7 RNA polymerase (50 unit/μL) and TTh RNaseH (10 unit/μL) were obtained from Toyobo (Osaka, Japan). Oligonucleotides were synthesized by Sigma-Aldrich Japan (Tokyo, Japan). The sequence of the oligonucleotides are as follows: non-template strand, 5′-TTTTTTAATACGACTCACTATAGGGATCTTCAGACACACGCTTGCATAGTTTTGCTTTG-3′; template strand, 5′-CAAAGCAAAACTATGCAAGCGTGTGTCTGAAGATCCCTATAGTGAGTCGTATTAAAAAA-3′; molecular beacon probe, 5′-[FAM]-CTATGCAAGCGTGTGTCTGAAGATGCATAG-[BHQ1]-3′. The T7 promoter is underlined. BHQ1 refers to black hole quencher version 1.

2.2. Vesicle preparation by PSGH

In PSGH, vesicle preparation is initiated by formation of a lipid/sugar film, followed by hydration with a buffer solution. To form the film, 20 μL of 10 mM DOPC in $CHCl_3$, 2 μL of 1 mM DOPE-PEG_{2000} in $CHCl_3$, 40 μL of 50 mM D-fructose in MeOH, 178 μL of $CHCl_3$, and 60 μL of MeOH were mixed. The amount of PEGylated lipid was 1 mol% of DOPC, which is the same ratio used by Yamashita et al. to prepare GUVs in a buffer containing monovalent ions only [23]. The amount of sugar was 10 × DOPC, as used by Tsumoto et al. [24]. The solution was put into a 10 mL round-bottom glass flask. The organic solvent was removed with a rotary evaporator (N-1000, EYELA, Japan) equipped with a vacuum pump (DIVAC, ULVAC, USA). The evaporator was set at a speed of 180 rpm, exhaust rate of 1.2 L/min, and temperature of 40 °C. After evaporation for five minutes, a lipid/sugar film of diameter ~2 cm is formed at the bottom of flask. To remove residual solvent, the flask was placed for 17 h in a vacuum desiccator set at 10 mmHg and room temperature. To hydrate, 2 mL of a solution containing 10 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 10 mM $MgCl_2$ was heated to 37 °C and gently poured into the flask. The flask was then sealed and incubated at 37 °C for 2 h to form vesicles. A sample of 1 mL was taken, and filtered through 40 μm mesh nylon (Cell strainer, BD Falcon, USA).

2.3. Preparation of GV reference

To determine lamellarity of thin-walled GVs, a reference mixture containing uni-, bi-, and trilamellar GVs was prepared according to Yamashita's method [23]. The composition of lipids in the reference was identical to that of vesicles prepared by PSGH to enable a direct quantitative comparison of fluorescence intensity. Thus, the reference mixture was prepared from a lipid film consisting of DOPC and DOPE-PEG_{2000} (1 mol% of DOPC), which was gently hydrated with deionized water for 2 h at 37 °C, and then filtered through 40 μm mesh nylon.

2.4. Analysis of synergism between PEGylated lipid and sugar

To test whether PEGylated lipid and sugar synergistically improve GUV productivity, two vesicle samples were prepared identically, except that either PEGylated lipid or sugar was excluded during preparation. Thus, a lipid film of DOPC (0.67 mM), doped either with PEGylated lipid (1 mol% of DOPC) or sugar (10 × DOPC), was hydrated at 37 °C for 2 h with buffer containing 10 mM Tris–HCl (pH 8.0), 100 mM NaCl, and 10 mM $MgCl_2$.

2.5. Fluorescent staining

All the vesicle membranes were stained with Nile red, which is a lipophilic red fluorescent dye. Nile red dissolved in $CHCl_3$ was added to phospholipid mixtures at 0.2 mol% of DOPC. To stain the encapsulated aqueous pool, Mag-Fluo-4, a hydrophilic green fluorescent dye was added at a final concentration of 1 μM to hydration buffers. The concentration of Nile red was the same as that used by Akashi et al. [25] to determine the lamellarity of thin-walled GVs. The concentration of Mag-Fluo-4 was a manufacturer-recommended one, at which the fluorescence intensity is proportional to the dye concentration.

2.6. Microscopy

Vesicle samples were placed between two cover glass slides, sealed with FrameSeal (Invitrogen, Carlsbad, CA, USA) and characterized with a phase contrast and fluorescence microscope (IX71, Olympus, Japan) equipped with a 20 × objective lens and a CCD camera (Model C4742-95-12ER, Hamamatsu Photonics, Japan). Red and green fluorescence images were obtained using corresponding filter and dichroic mirror units (WIG, excitation