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Inward multivesiculation at the basal membrane of adherent giant phospholipid vesicles

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ABSTRACT

Adherent giant vesicles composed of phosphatidylcholine, phosphatidylserine and biotinylated lipids form clusters of inward spherical buds at their basal membrane. The process is spontaneous and occurs when the vesicles undergo a sequence of osmotic swelling and deswelling. The daughter vesicles have a uniform size (diameter ≈ 2–3 μm), engulf small volumes of outer fluid and remain attached to the region of the membrane from which they generate, even after restoring the isotonicity. A pinning–sealing mechanism of long-wavelength modes of membrane fluctuations is proposed, by which the just-deflated vesicles reduce the surplus of membrane area and avoid excessive spreading and compression via biotin anchors. The work discusses the rationale behind the mechanism that furnishes GUVs with basal endovesicles, and its prospective use to simulate cellular events or to create molecular carriers.

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1. Introduction

Giant lipid vesicles are getting more and more prominent. Not only do they provide free-standing artificial membranes where single biomolecules can be placed at will and tested [1], but they also provide a scenario devoid of chemical and structural complexity, in which to recreate the synthesis of proteins [2,3], the curvature-induced sorting of membrane-active molecules [4,5], the dynamics of the cytoskeleton [6, 7], cell adhesion [6,8,9] or endocytosis [10]. Furthermore, they can act as cellular mimics [11]. They exhibit a dynamics of their own that resembles, and contributes to understand, aspects of the cell activity, the likely morphology and behaviour of primitive cells. Under the appropriate conditions, liposomes can fuse [12], generate nanotubes or buds out of their membranes [13,14,15,16,17], develop rudimentary ways to compartmentalize their interior [16,17], and even compete for resources as in the case of fatty acid vesicles [18]. This type of behaviour has arisen the question as to whether cells profit from the intrinsic properties and dynamics of their carcass [9,10]. Hence the study on the dynamics and shape of vesicles, though extended over decades, does not cease to inspire biosynthetic approaches. These include giant vesicle-based models to investigate the mechanisms of host-invasion by bacteria or viruses [19,20,21], the effect of antimicrobial peptides and toxins [22,23], and macroautophagy [24], as well as methods for the fabrication of giant liposomes with enhanced biomimetic features [25,26,27,28].

Here evidence is shown of inward budding in osmotically deflated giant liposomes when the latter are proximal to an adhering layer and have undergone a sequential osmotic swelling–deswelling. The liposomes are made of phosphatidylcholine, phosphatidylserine, and biotinylated-phosphatidylethanolamine lipids. The latter are molecular anchors that render the vesicles adherent to surfaces functionalized with streptavidin. The daughter vesicles, of spherical shape and uniform size (2–3 μm in diameter) appear in planar, raspberry-like clusters *only* at the basal membrane (i.e. adjacent to the substrate) of the parent vesicle. The fact that the vesicles enclose sub-femtoliter volumes of outer fluid and hardly move, suggests that they form by invaginations of the basal membrane. They remain attached to the parent vesicle, even after reverting to isotonic conditions. As far as the author knows, this is the first report on anisotropic vesiculation inside giant liposomes with cholesterol-free membranes that contain molecular anchors. The work invites to reconsider the role of osmotic history, membrane fluctuations, lateral tension and adhesion in the morphology of adherent vesicles.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl) (sodium salt, DOPE-cap-biot) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt, DOPE-Rhod) were used as received (Avanti Polar Lipids, Alabaster, AL, USA), dissolved in HPLC-grade chloroform (Carl Roth), aliquoted and stored in sealed vials under N_2 until further use. Poly(L-lysine) (PLL, 20 kDa) grafted with poly(ethylene glycol) (PEG, 2 kDa) and biotinylated poly(ethylene

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glycol), (17% Biotin-PEG 3.4 kDa), (biot-PEG-g-PLL, SuSoS, Switzerland). Phosphate buffer saline (PBS, Fluka) with 8 mM sodium hydrogen phosphate, 2 mM potassium dihydrogen phosphate, 3 mM potassium chloride, and 137 mM sodium chloride, was employed for the vesicle formation and the adsorption of the polymers on coverslips. Enhanced green fluorescent protein (EGFP) was expressed in *Escherichia coli* out of plasmid vector pET28a and purified by gel permeation chromatography as reported elsewhere [29].

2.2. Formation of giant vesicles and encapsulation of EGFP

Giant vesicles were formed by electroswelling in a self-made perfusion cell, consisting of two parallel-plate glass-ITO electrodes (25×25 mm^2, 8–12 Ω, SPI Supplies, PA, USA) and a 2 mm thick PDMS spacer perforated in a hole of approximately 12 μm in diameter. 4–5 μl of a solution of 0.8 μmol POPC, 0.1 μmol DOPS, 0.089 μmol DOPE-cap-biot and 0.01 μmol of DOPE-Rhod in chloroform (final concentration 1 μmol/ml) was drop-cast on the lower electrode and let dry in a desiccator overnight. PC and PS lipids, two of the major lipid components in mammalian plasma membranes [30], were mixed in a ratio that has been previously reported to avoid vesicle deformation or fusion upon immobilization [31]. The ITO electrodes were previously sonicated in chloroform (15 min), in a 2% aqueous solution of SDS (30 min), rinsed in Milli-Q water and absolute ethanol, and heated at 70 °C for 50 min (50 min) [32]. The electroformation cell was sealed and filled with a PBS solution containing EGFP and sucrose in a concentration of 0.1 mg/ml and 0.1 M, respectively. Electroswelling proceeded by the application of an AC potential of 500 Hz across the electrodes with a function generator (GF-857,Promax, Spain). First, the amplitude was increased from 0 to 3.6 V (peak-to-peak voltage) in the first 30 min, and kept at 3.6 V for 90 min. The medium in the chamber was then replaced with EGFP-free solution of 0.1 M sucrose. After exchanging the medium, the AC frequency was gradually decreased from 500 Hz to 50 Hz at a rate of 75 Hz every 10–15 min and kept at 50 Hz for another 20 min before completion. The frequency and amplitude of the electric potential were continuously monitored with an oscilloscope (DSO3062A, Agilent, USA). After gentle tapping to detach the vesicles from the electrode, the dispersion was transferred to a sterile test tube and kept at room temperature until further use.

Substrate functionalization was conducted under a laminar flow hood on freshly cleaned circular coverslips of borosilicate glass (24 mm diameter, Menzer-Glässer, Germany) mounted in a commercial well-like holder with a volume capacity of approximately 500 μl and optimised for optical microscopy (CoverSlip Holder®, JPK Instruments, Germany). Previously, the glass coverslips were immersed in 2% Hellmanex and sonicated for 30 min. After rinsing thoroughly with ultrapure water (18.6 MΩ cm, Milli-Q, Millipore, MA, USA) the coverslips were gently blown dry with N_2 before being placed in the holder. The coverslips were incubated first in a PBS solution of biot-PEG-g-PLL (300 μl, 1 mg/ml) and after in a PBS solution of streptavidin (300 μl, 0.1 mg/ml) for at least 40 min. Between incubations, the coverslips were rinsed at least thrice in PBS and after the second incubation, in a PBS solution of glucose (0.1 M).

2.3. Sample preparation for microscopy

30 μl of the vesicle dispersion in PBS-sucrose (0.1 M) was gently added in the holder bottomed with the coated coverslip filled with an isomolar solution of PBS-glucose (300 μl). Experiments usually started after a 15–30 min lapse to let the vesicles sink and adsorb on the substrate.

The osmolarity of the outer medium was changed by varying the concentration of NaCl in the PBS buffer from 137 mM (isotonic solution, total ion concentration = 150 mM), down to 37 mM (hypotonic solution, total ion concentration = 50 mM), or up to 187 mM (hypertonic solution, total ion concentration). The concentration of glucose was fixed to 0.1 M in all cases.

2.4. Determination of vesicle height

Vesicle heights were determined from force curves with a scanning probe microscope (Nanowizard 3®, JPK Instruments, Germany) equipped with a sample-moving stage (CellHesion®, JPK Instruments, Germany) for large vertical displacements. Both the SPM and the stage were mounted on the inverted microscope to ease the localization of the vesicles and probe positioning. Triangular cantilevers with spring constants in the range of 0.014–0.023 N/m (MLCT, Bruker, Camarillo CA) and silica beads glued at their free ends (8 μm diameter, μ-particles, Germany) were employed as probes. The sample was approached to and withdrawn from the probe at 5 μm/s. Force curves were acquired as a function of the vertical position Z of the sample, namely the substrate and the vesicle apex. The positions at which the substrate and the vesicle contact the probe, $Z_{0,s}$ and $Z_{0,v}$ respectively, were determined from the corresponding curves by visual inspection and used to calculate the vesicle height, h, as $h = Z_{0,v} - Z_{0,s}$.

2.5. Visualization of vesicles (light microscopy)

The microscopic observations were carried out with an inverted fluorescence microscope (AxioObserve D1, Zeiss, Germany) equipped with an aperture correlation module (Vivatome®, Zeiss, Germany) for optical sectioning. Optical micrographs were acquired at room temperature, with a plan-apochromat objective (63×/1.4 NA oil immersion, Zeiss, Germany) and a sideport-connected, high-resolution camera (AxioCam MRm, Zeiss, Germany). Narrow band-pass excitation and emission filters were employed for conventional red and green emission fluorescence (filter sets 43 and 38, Zeiss, Germany). For optical sectioning, a DAPI/FITC/Rhod filter module (Zeiss, Germany) was employed. Both setups operated with two independent, white-light excitation sources and light guides (HXP 120C, Zeiss, Germany). Images were acquired and contrast-enhanced with a commercial image acquisition software (ZEN, Zeiss, Germany). Vesicle diameters and adhesion areas were calculated from the as-obtained micrographs using the outline tools and measure options of ImageJ (http://imagej.nih.gov/ij/).

3. Results and discussion

Vesicles composed of POPC (80% mol) and DOPS (10% mol), doped with the fluorescent lipid (DOPE-Rhod, 1% mol) and the biotinylated lipid anchor (DOPE-cap-Biot, 8.9% mol) are deposited on a substrate with biotin-linked streptavidin, in a medium of lower density but equal osmolality. As a result of the density difference, the vesicles readily sink to the substrate, a glass coverslip coated with a layer of PLL grafted with chains of PEG (see scheme in Fig. 1a). 17% of these chains are biotinylated at their free chains. On a hydrophilic surface such as glass, SiO_2 or TiO_2 the PLL-g-PEG molecules have been found to adopt a comb-like conformation, where the PLL backbones lie parallel to the surface and the PEG grafts orient upright in a brush-like conformation [33] The latter renders the biotin moieties accessible to bind to streptavidin molecules. These in turn bind to the sinking vesicles through the lipid anchors. Then the adhered vesicles undergo a sequence of osmotic events (Fig. 1b) that lead first to swelling in a hypotonic medium, followed by deswelling in a hypertonic medium for periods of at least 30–60 min.

Under the fluorescence microscope and in isotonic and hypotonic media, the morphology of an adhered giant vesicle resembles that of a spheroidal cap as Fig. 1c and d shows. The bound region, or the membrane area adhered to the substrate, appears flat and homogeneously fluorescent. The observation gives thus no visual indication of domain formation or phase separation occurring at scales larger than those imposed by the spatial resolution of the microscope. Decreasing the ionic

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