Colloids and Surfaces B: Biointerfaces 136 (2015) 545–552

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Thermally-induced aggregation and fusion of protein-free lipid vesicles

Maitane Ibarguren[a,1], Paul H.H. Bomans[b], Kepa Ruiz-Mirazo[a,c], Peter M. Frederik[b], Alicia Alonso[a], Félix M. Goñi[a,*]

[a] Unidad de Biofísica (Centro Mixto CSIC, UPV/EHU), and Departamento de Bioquímica, Universidad del País Vasco, Apto. 644, 48080 Bilbao, Spain
[b] Soft Matter CryoTEM Research Unit, Laboratory for Materials and Interface Chemistry, P.O. Box 513, 5600MB Eindhoven, The Netherlands
[c] Logic and Philosophy of Science Department, University of the Basque Country, Spain

ARTICLE INFO

Article history:
Received 3 June 2015
Received in revised form 21 August 2015
Accepted 24 September 2015
Available online 30 September 2015

Keywords:
Fusion
Cholesterol
Non-lamellar phases
Thermal fluctuations

ABSTRACT

Membrane fusion is an important phenomenon in cell biology and pathology. This phenomenon can be modeled using vesicles of defined size and lipid composition. Up to now fusion models typically required the use of chemical (polyethyleneglycol, cations) or enzymatic catalysts (phospholipases). We present here a model of lipid vesicle fusion induced by heat. Large unilamellar vesicles consisting of a phospholipid (dioleoylphosphatidylcholine), cholesterol and diacylglycerol in a 43:57:3 mol ratio were employed. In this simple system, fusion was the result of thermal fluctuations, above 60 °C. A similar system containing phospholipid and cholesterol but no diacylglycerol was observed to aggregate at and above 60 °C, in the absence of fusion. Vesicle fusion occurred under our experimental conditions only when ^{31}P NMR and cryo-transmission electron microscopy of the lipid mixtures used in vesicle preparation showed non-lamellar lipid phase formation (hexagonal and cubic). Non-lamellar structures are probably the result of lipid reassembly of the products of individual fusion events, or of fusion intermediates. A temperature-triggered mechanism of lipid reassembly might have occurred at various stages of protocellular evolution.

1. Introduction

Membrane fusion occurs in cells whenever two vesicles coalesce giving rise to a single compartment. The "vesicles" may be two liposomes, two cells (e.g., sperm and ovum), one virus and one cell (e.g., HIV particle and T-lymphocite), or one intracellular vesicle and the plasma membrane (e.g., in the release of neurotransmitters), as examples of the multitude of membrane fusion events. In spite of the obvious biological interest and of abundant studies, the molecular mechanisms of membrane fusion are not completely understood. One debated point is the relative importance of the roles of lipids and proteins in the fusion event [1]. Several examples are known of fusion in pure lipid vesicles, but in all cases addition of a chemical or a biological catalyst was required [2–6]. Early [7,8] and more recent [9] studies have shown that polyethyleneglycol induces fusion of pure lipid vesicles by creating an osmotic force that drives membranes into closer contact in a dehydrated region. It is however disputable the extent to which these experiments mimic the physiological conditions in which free water exists. Many efforts have been devoted to characterizing proteins that mediate fusion [10,11]. However, we might not be able to determine the mechanism of those proteins until we understand what happens to two adjacent lipid bilayers to make them fuse. Once the mechanism of fusion in pure lipid systems is understood, the various ways by which proteins catalyze these processes in the cell may be more easily identified.

Cholesterol (Chol) is found in all mammalian cells; the human erythrocyte plasma membrane contains as much as 45 mol% Chol [12]. The physiological significance of this molecule is not clear, but many studies show that it alters a number of membrane physicochemical properties [13], and appears to play a direct role in cellular fusion [14]. Chol is found to destabilize phosphatidylethanolamine (PE) and phosphatidylcholine (PC)-PE bilayers, inducing the formation of inverted hexagonal (H_{II}) phase in these systems [15,16]. It also reduces the amount of diacylglycerol required to induce the lamellar-hexagonal transition [17]. Chol has a paradoxical effect

* Corresponding author at: Unidad de Biofísica (CSIC, UPV/EHU) and Departamento de Bioquímica, Universidad del País Vasco, Barrio Sarriena s/n, 48940 Leioa, Spain. Fax: +34 946013360.
E-mail addresses: maitaneibarguren@hotmail.com (M. Ibarguren), p.h.h.bomans@biomat.tue (P.H.H. Bomans), kepa.ruiz-mirazo@ehu.es (K. Ruiz-Mirazo), peter.frederik@elmi.unimaas.nl (P.M. Frederik), alicia.alonso@ehu.es (A. Alonso), felix.goni@ehu.e (F.M. Goñi).
[1] Present address: Laboratory of Molecular Cell Biomedicine, University of the Balearic Islands-Lipopharma Therapeutics, S L. Palma, Spain.

http://dx.doi.org/10.1016/j.colsurfb.2015.09.047
0927-7765/

in cell membranes: it has the ability to form rigid, low-curvature raft-like patches, while still being able to promote formation of highly-curved, nonlamellar inverted hexagonal and cubic phases [18]. Chol also induces lateral phase separation by forming liquid-ordered domains separated from regions enriched in unsaturated PCs [19,20]. Control of the extent of lateral phase separation of unsaturated PCs may be one mechanism that cells use to modulate the spatial and temporal occurrence of fusion events [18].

Lipid polymorphism plays an essential role in membrane fusion [21]. Even if lipids in cell membranes adopt the disposition of lamellar phases [22], the mechanism of fusion of two apposed lipid bilayers necessarily requires the formation of a transient non-lamellar intermediate. This intermediate has been called the "stalk" [4,23–29]. Siegel et al. [30] emphasized the linkage between formation of "isotropic phase" and fusion, and proposed a causal relationship between the two. Nieva et al. [31] suggested a bicontinuous inverted cubic (Q_{224}) phase structure formed by fusogenic lipid mixtures containing diacylglycerols (DAG). DAG is a powerful agent in restructuring lipid bilayers and cell organelles [32–34]. Low levels of DAG are surprisingly effective at stabilizing inverted phases in phospholipid systems [30,35–38]. DAG stabilizes inverted phases mainly by reducing (making more negative) the spontaneous radius of curvature of the membrane [39–41].

Searching for the simplest fusion model system, in the absence of any added catalyst, we aimed to fuse protein-free unilamellar vesicles of a defined lipid composition by the sole effect of thermal motion. In a recent work, two adjacent vesicles were heated by laser irradiating gold nanoparticles, this caused fusion with associated membrane and cargo mixing [42]. We observed that vesicles composed of a single phospholipid, Chol and DAG (at a 43:57:3 mol ratio) indeed fuse at temperatures above 60 °C in the absence of protein. Chol promotes thermally-induced vesicle aggregation, while DAG is essential in the generation of inverted non-lamellar lipid phases and in the process of membrane fusion.

2. Materials and methods

2.1. Materials

1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and Chol were purchased from Avanti Polar Lipids (Alabaster, AL). Egg DAG was purchased from Lipid Products (South Nutfield, UK). DAG fatty acid composition was C16:0, 32%; C18:0, 12%; C18:1, 36%; C18:2, 13%; other, 7%. *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE) and rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Rho-PE) were supplied by Molecular Probes, Inc. (Eugene, OR). A kit for measuring cholesterol concentration was supplied by BioSystems (Barcelona, Spain).

2.2. Multilamellar vesicle (MLV) preparation

For MLV preparation the lipids were dissolved in chloroform/methanol (2:1) and mixed in the required proportions, and the solvent was evaporated to dryness under a stream of nitrogen. Traces of solvent were removed by evacuating the samples under high vacuum for at least 2 h. The dry lipids were hydrated in 25 mM HEPES, 150 mM NaCl, pH 7.4 helping dispersion by stirring with a glass rod. The lipid concentration of the dispersions was 10% wt/wt. To ensure homogeneous dispersion the hydrated samples were pushed between two syringes through a narrow tubing (0.5 mm internal diameter, 10 cm long) 100 times at 45 °C.

2.3. Large unilamellar vesicle (LUV) preparation

LUV of diameters 100–150 nm were prepared from MLV by the extrusion method [43] using Nuclepore filters 0.1 μm pore diameter at room temperature, in 25 mM HEPES, 150 mM NaCl, pH 7.4. The final phospholipid concentration was measured in terms of lipid phosphorous [44] and the Chol amount was assayed with a commercial kit based on three coupled reactions using Chol esterase, Chol oxidase and peroxidase. When Chol and phospholipid were initially mixed at a 2:1 mol ratio, the bilayer composition after LUV formation was 57 ± 6.4 Chol and 43 ± 4.1 phospholipid. Note that, as shown by Ibarguren et al. [45] final Chol concentrations in extruded LUV may be different from the ones in the starting mixture. Throughout this paper the 43:57 DOPC:Chol mol ratio refers to measurements after LUV formation. Addition of up to 3 mol% DAG to the 43:57 mixture did not change the effective phospholipid:Chol ratio.

2.4. Thermal treatment, aggregation and lipid mixing measurements

A stock of buffer was pre-incubated at the desired temperature in a water bath. Then 550 μl buffer were added to a cuvette in a thermostatted setting and again left to equilibrate until the selected temperature was reached and stabilized. LUVs (≈100 μl, final concentration 0.3 mM) were added at time 0 to the buffer. Addition of this LUV volume did not cause any detectable change in the temperature of the system. For all thermal treatments the spectroscopic cuvettes were covered by a lid. No measurable evaporation occurred during the experiments. Liposome aggregation was estimated as an increase in turbidity (absorbance at 400 nm) [46], measured as a function of time in a Uvikon 922 (Kontron Instruments, Milan, Italy).

Lipid mixing was assayed by the resonance energy transfer method [47] using NBD-PE and Rho-PE. Vesicles containing 2% NBD-PE and 2% Rho-PE in their bilayer composition were mixed with probe-free liposomes at a 1:4 ratio. NBD emission was followed at 530 nm (excitation wavelength at 465 nm with a cut-off filter at 515 nm). 100% mixing was set after addition of 1 mM Triton X-100.

Lipid bilayer fusion was assayed as mixing of inner monolayer lipids as described by Montes et al. [48]. For this assay vesicles containing 2% NBD-PE and 2% Rho-PE are briefly treated with sodium dithionite, a reagent that does not penetrate the membrane and that bleaches the fluorescence of the probes located in the outer monolayers. The resulting vesicles, containing fluorescent probes only in the inner monolayers are mixed with probe-free liposomes at 1:4 ratio and lipid mixing is monitored following standard procedures [47]. Fluorescence measurements were performed in an Aminco Bowman Series 2 luminescence spectrometer.

2.5. Vesicle contents release and leakage mechanism

Vesicle efflux was measured with the ANTS:DPX system as in [47,48]. A series of measurements was carried out to find out whether partial leakage occurs as a result of an all-or-none event (some of the vesicles release all of their contents) or as a graded event (all of the vesicles release part of their contents). Briefly, a quench curve for vesicles containing varying concentrations of ANTS and DPX, always at a 3.6/1 mole ratio, was constructed. Then the extent of leakage induced by thermal fluctuations was measured at various temperatures, keeping constant lipid concentration. Samples were applied to a Sephadex G-75 column to separate the lipid vesicles; fluorescence of the eluted vesicle fraction was measured before and after detergent solubilization, and