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Morphology and Fine Structure of Membranes upon Osmotic Upshifts

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Lipid bilayers are impermeable to most polar molecules. Osmoregulated transporters are responsible for controlling the intracellular osmolarity and protecting the cell against changes in osmolality in the environment. The mechanisms by which membranes regulate the activity of these transporters are still largely unknown. In this paper we investigate the response to hyperosmotic stress in artificial, chemically well-defined membrane models called Giant Unilamellar Vesicles (GUV). The lipid compositions analysed are relevant for the activity of an ABC-transport system that is controlled by the physicochemical properties of the membrane bilayer. Morphology changes are monitored by phase-contrast optical microscopy, and fine structural details related to domain formation are investigated by fluorescence confocal optical microscopy.

KEY WORDS: Confocal microscopy; osmotic stress; giant unilamellar vesicle; transporter; phosphatidylglycerol.

INTRODUCTION

Cell membranes serve as a barrier to the passage of most ions and big molecules, owing to the hydrophobic interior of phospholipid bilayer. This barrier function allows the cell to keep the solute concentrations in the cytosol relatively constant and different from those in the extracellular fluid. When the cell is exposed to environments with too low or too high osmolality, morphological and structural changes in the membrane occur that activate a number of transporters and channels [1,2]. This prevents dramatic changes in cell volume and protects the cell from lysing.

Although the physical mechanisms by which membrane components regulate the functioning of embedded proteins are not fully understood, it is evident that different lipid compositions, in particular associated with the presence of anionic lipids, affect transport systems, such as osmoregulated transporters [2–4]. Therefore, it is important to have a better insight into how membranes respond to osmotic stress and how their response changes with the lipid composition. From this point of view, Giant Unilamellar Vesicles (GUV) [5] offer a good membrane model as they provide spherical closed bilayers with a well-defined chemical composition. Compared to submicroscopic vesicles, GUV are more responsive to membrane asymmetries created by environmental factors such as osmolality changes [6]. Finally, GUV are suitable artificial membranes for optical microscopy.

In this paper we present the first results of a characterization of GUV response to transmembrane osmotic gradients by analysing their shape and morphology by employing phase-contrast microscopy, and their membrane fine structure and domain formation by fluorescence confocal microscopy. Lipid compositions used are

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relevant for the activity of an osmoregulated ABC transporter in *Lactococcus lactis* [2]. A more extensive study will be published in a future article.

EXPERIMENTAL

Chemicals

L-α-Dioleoyl-phosphatidylcholine (DOPC), L-α-dioleoyl-phosphatidylethanolamine (DOPE), L-α-dioleoyl-phosphatidylglycerol (DOPG), and L-α-oleoyl-lauroyl (*N*-NBD)-phosphatidylglycerol (*N*-NBD-PG) were purchased from Avanti Polar Lipids. All other reagents were of analytical grade.

Preparation of Giant Unilamellar Vesicles (GUV)

Giant unilamellar vesicles were prepared by the electroformation technique [5]. With this approach, GUV are produced with a size varying from 10–100 μ*M*. The chamber for vesicle preparation was composed of two microscope slides, each coated with an optically transparent and electrically conductive thin layer of indium tin oxide (ITO). Lipids (DOPG/DOPC/DOPE, 38:12:50 or 0:50:50 molar ratio) in chloroform/methanol (9:1) were deposited on the ITO glass plates, and the solvent was evaporated under vacuum. The sealant paste Sigillum wax (Vitrex, Denmark) was used as a spacer between the two plates. After adding water into the chamber (∼300 μL), a voltage of 1,1 V at 10-Hz frequency was applied for 2–3 h through thin metal electrodes sealed on the glass plates. In some experiments, a flow chamber (Warner Instruments RC-21) was used to gradually impose an osmotic gradient to the GUV, thereby avoiding vesicle collapse.

Phase-Contrast Microscopy

Morphological changes upon osmotic shifts were monitored by phase-contrast microscopy, using an Axiovert S100 TV Zeiss microscope, equipped with a Zeiss CP-Achromat 40×, Ph 2 objective and a CCD camera (Hamamatsu C5985).

Fluorescence Confocal Imaging

Confocal images were obtained by focusing the excitation light of an Ar ion laser (Coherent) at 488 n*m* into a 15-μm pinhole spatial filter. The spatially filtered light was deflected by a dichroic mirror (500 DCLP) and focused by the objective (Zeiss NeoFluor 40×, NA = 0.75) in an inverted microscope (Axiovert S100 TV, Zeiss) into the sample. A 15-μ*M* pinhole in front of the detector, an avalanche photodiode (EG & G, Canada) eliminated out-of-focus contributions, allowing a confocal sectioning of the sample. An OG515 filter filtered out the scattered excitation light. The excitation power was kept low to prevent photobleaching (1 μW).

RESULTS AND DISCUSSION

The goal of the present work was to describe the response of GUV to osmotic stress under experimental conditions that are relevant for the functioning of osmoregulated machineries, such as the ABC transporter OpuA in *L. lactis* [2]. The lipid composition used for membrane reconstitution of OpuA consisted of phosphatidylglycerol (PG), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) and mimicked the composition of the native membrane of *L. lactis* in terms of fraction of bilayer vs. non-bilayer lipids, anionic versus neutral lipids, and lipids capable of forming hydrogen bonds with proteins.

The GUV morphology for different lipid compositions and upon different osmotic gradients was monitored by phase-contrast microscopy. Although prepared under identical conditions, the GUV morphologies were highly dependent on the lipid compositions. In Fig. 1a a phase-contrast image of GUV (DOPG/DOPC/DOPE 38:12:50 molar ratio) prepared in water is shown: typical shapes are ovoidal, but vesicles inside one another and a high percentage (20%) of multilamellar vesicles are also observed. A statistical analysis of the size distribution of GUV is shown in Fig. 1b. Liposomes reported at 70 μ*m* consist of fiber-like structures. In the absence of PG, GUV composed of DOPC/DOPE (50:50 molar ratio) and prepared in water yielded spherical and mainly unilamellar (>99%) vesicles (Fig. 1c). Fiber-like structures were totally absent, and spherical vesicles could reach large average diameters up to ∼70 μ*m* (Fig. 1d).

Clear differences in the response to osmotic upshifts were observed for different lipid compositions. In the presence of PG, shape modifications occured and obloid giant vesicles were transformed into smaller dumbell-shaped (8-shaped) or elongated fibers (Fig. 2a, size distribution in Fig. 2b). The final morphology is independent of the osmotic gradient applied (10 m*M*–300 m*M* KCl gave qualitatively identical shape changes). If no PG was included in the GUV (DOPC/DOPE 50:50), the vesicles, mainly spherical in water, were transformed into flaccid obloid liposomes and then into spheres of smaller size upon hyperosmotic shock (data not shown). This type

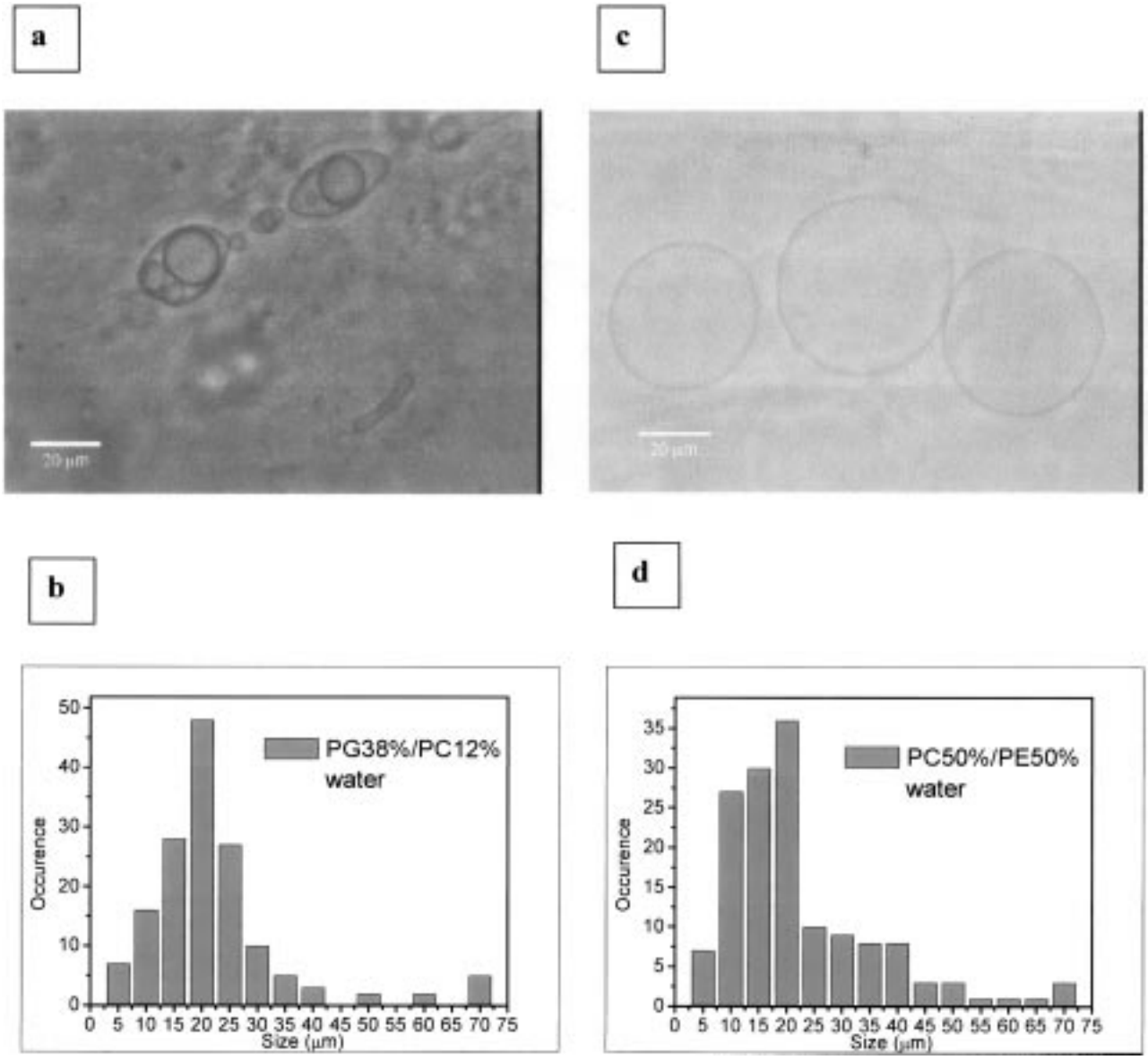

Fig. 1. (a) Phase-contrast image of GUV (DOPG/DOPC/DOPE 38:12:50 molar ratio) in water. (b) Size distribution of a population of GUV (DOPG/DOPC/DOPE 38:12:50 molar ratio) in water. (c) Phase-contrast image of GUV (DOPC/DOPE 50:50 molar ratio) in water. (d) Size distribution of a population of GUV (DOPC/DOPE 50:50 molar ratio) in water. A Zeiss CP-Achromat 40×, Ph2 objective was used. The statistical analysis was carried out for ~200 vesicles, and observations from three independent experiments were used. Liposomes with a diameter below 5 μ*M* were not counted.

of transformations are similar to those given by GUV composed of egg phosphatidylcholine [6].

Fluorescence confocal imaging was used to characterize the fine structure of the bilayers. By using a fluorescent probe with a PG head-group, we investigated the possibility that PG segregation occurs in flat regions of particular bilayer structures. For the lipid compositions analysed, we did not observe any tendency of PG to accumulate in regions with particular curvature in the plane of the membrane, independent of the morphology of the vesicles.

However, an inhomogeneous distribution of PG molecules in the plane of the GUV membrane was observed in iso-osmotic conditions (pure water; Fig. 3a), while the PG molecules appeared to be homogeneously distributed after the osmotic upshift (Fig. 3b). In the absence of PG, confocal images of GUV showed a homogeneous distribution of fluorescent lipids in the plane of

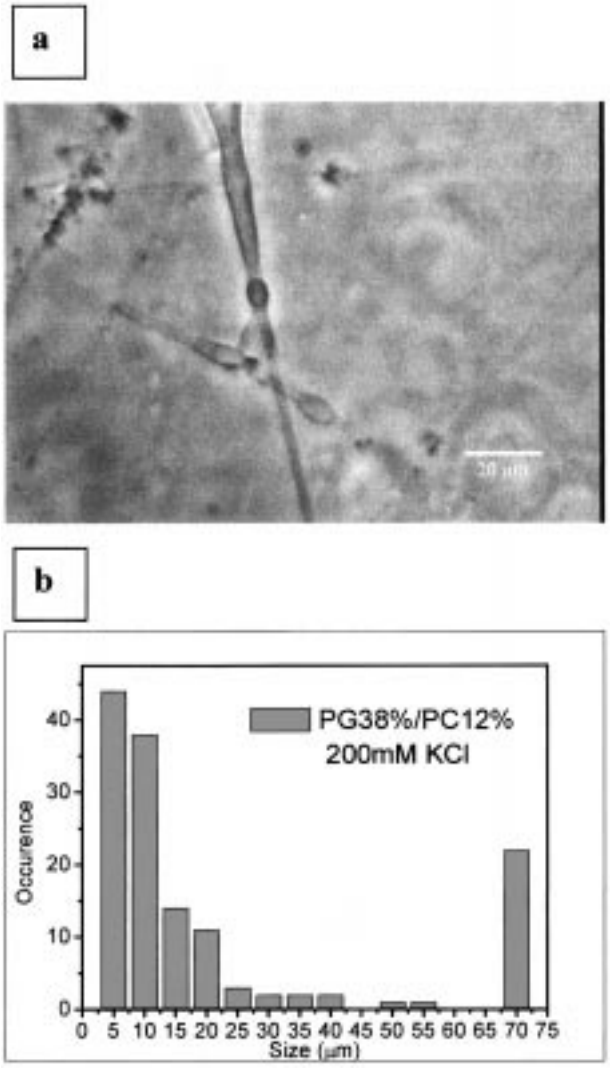

Fig. 2. (a) Phase-contrast image of GUV (DOPG/DOPC/DOPE 38:12:50 molar ratio) after an osmotic shock imposed by a 200-m*M* KCl buffer. (b) Size distribution of a population of GUV (DOPG/DOPC/DOPE 38:12:50 molar ratio) after osmotic shock. A Zeiss CP-Achromat 40×, Ph2 objective was used. The statistical analysis was carried out for ~200 vesicles and observations from three independent experiments were used. Liposomes with a diameter below 5 μ*M* were not counted.

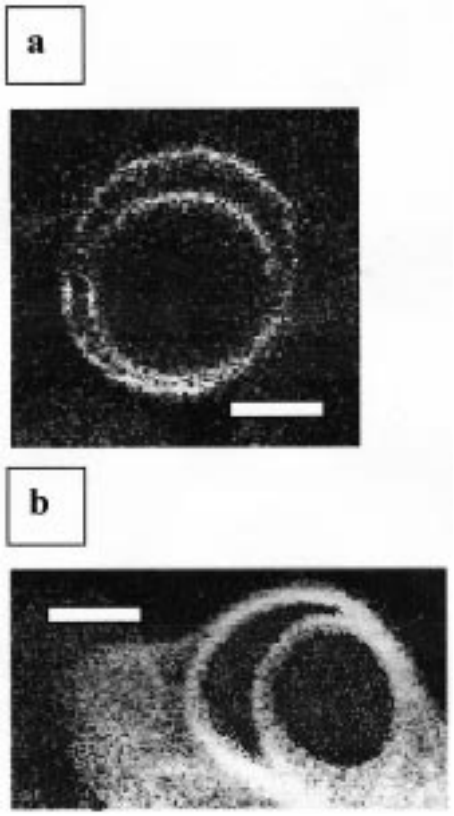

Fig. 3. (a) Fluorescence confocal images of GUV (DOPG/DOPC/DOPE 38:12:50 molar ratio, 0.1% *N*-NBD-PG) in pure water. (b) Fluorescence confocal images of GUV (DOPG/DOPC/DOPE 38:12:50 molar ratio, 0.1% NBD-PG) after an osmotic upshift imposed by 200 m*M* KCl. The excitation beam (λ_{ex} 488 nm) was focused onto the sample with a Zeiss NeoFluor 40×, NA = 0.75 objective, and the fluorescence was detected by an avalanche photodiode with a 15-μ*M* pinhole Bar = 10 μ*M*.

the membrane (data not shown) in iso-osmotic conditions as well as hyperosmotic conditions.

CONCLUSION

In this work we investigated osmotic stress in giant unilamellar vesicles (GUV). Monitoring of morphological changes upon osmotic shifts was complemented by fluorescence confocal imaging of GUV to observe the fine details of the lipid distribution in the plane of the membrane. To better understand how membranes regulate the activity of membrane transporters, the connection between the membrane response to environmental changes and the membrane composition at molecular level need further investigation.

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