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Segregative Clustering of Lo and Ld Membrane Microdomains Induced by Local pH Gradients in GM1-Containing Giant Vesicles: A Lipid Model for Cellular Polarization

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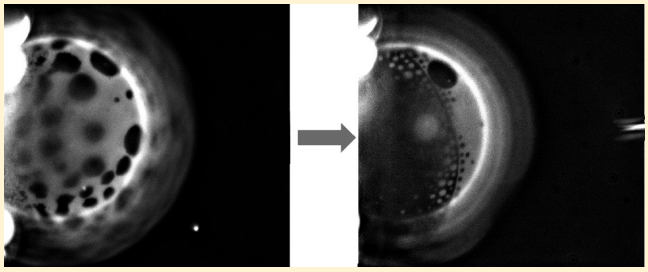
Supporting Information

ABSTRACT: Several cell polarization processes are coupled to local pH gradients at the membrane surface. We have investigated the involvement of a lipid-mediated effect in such coupling. The influence of lateral pH gradients along the membrane surface on lipid microdomain dynamics in giant unilamellar vesicles containing phosphatidylcholine, sphingomyelin, cholesterol, and the ganglioside GM1 was studied. Lo/Ld phase separation was generated by photosensitization. A lateral pH gradient was established along the external membrane surface by acid local microinjection. The gradient promotes the segregation of microdomains: Lo domains within an Ld phase move toward the higher pH side, whereas Ld domains within an Lo phase move toward the lower pH side. This results in a polarization of the vesicle membrane into Lo and Ld phases poles in the axis of the proton source. A secondary effect is inward tubulation in the Ld phase. None of these processes occurs without GM1 or with the analog asialo-GM1. These are therefore related to the acidic character of the GM1 headgroup. LAURDAN fluorescence experiments on large unilamellar vesicles indicated that, with GM1, an increase in lipid packing occurs with decreasing pH, attributed to the lowering of repulsion between GM1 molecules. Packing increase is much higher for Ld phase vesicles than for Lo phase vesicles. It is proposed that the driving forces for domain vectorial segregative clustering and vesicle polarization are related to such differences in packing variations with pH decrease between the Lo and Ld phases. Such pH-driven domain clustering might play a role in cellular membrane polarization processes in which local lateral pH gradients are known to be important, such as migrating cells and epithelial cells.



INTRODUCTION

It is now established that cellular plasma membranes are mosaics of different types of domains involving lipid or protein interactions.[1] During functional cellular processes, many of these domains undergo specific modulations of their sizes which can vary from hundreds of nanometers up to tenths of micrometers. Such dynamical lateral remodeling may culminate in phenomena termed "cell polarization", i.e., permanent or transient lateral compartmentalization of two or more compositionally and functionally distinct regions in the plasma membrane.[2]

Among the most documented types of microdomains are the so-called lipid rafts (i.e., liquid-ordered microdomains enriched in sphingolipid and cholesterol).[3] On the other hand, the validity of the raft concept has been questioned[4,5] and interrogations still exist concerning their size dynamics, heterogeneity, and asymmetry.[3] A more recently investigated type of microdomain is represented by ganglioside-specific microdomains. Gangliosides are glycosphingolipids that bear a protonable sialic acid moiety. These are present in lipid rafts and may also form distinct specific microdomains.[6−8] Both rafts and ganglioside-specific microdomains are involved in numerous cellular activation, transduction, and signaling functions. Cellular processes associated with raft/gangliosides microdomains often involve their clustering and aggregation, eventually contributing to cell polarization events. Two types of mechanisms have currently been mainly considered and documented as responsible for such microdomain aggregation:[9] (1) cross-linking of microdomain membrane components by multivalent extracellular ligands and (2) dragging of microdomains by cytoskeletal rearrangement through specific cytoskeleton−membrane interaction. A role of pH has not yet been considered. In eukaryotic cells, both external and cytoplasmic pH is globally uniform and near-neutral. However, there are specific situations where such pH uniformity may be locally and dynamically affected, particularly in juxtamembrane regions.[10] Many such situations are related to a nonuniform distribution or activation of the Na^+/H^+ exchangers

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(NHE).[11,12] NHE-dependent local pH heterogeneities or lateral gradients have been directly measured in oligodendrocytes,[13] migrating cancer cells,[14−16] and epithelial cells.[17,18] Interestingly some of the functional processes associated with such pH heterogeneities have been linked to protonation changes of acidic phospholipids, affecting either interactions with signaling proteins[19] or membrane curvature.[20] Until now, the relevance of a possible effect of pH on the dynamics, size, and lateral distribution of membrane domains containing acidic lipids (e.g., ganglioside-containing rafts or other microdomains) has not been considered. In several cell types, however, cell polarization is coupled to local pH heterogeneities. For instance, in the case of polarized migrating cells, NHE1 is necessary for migration[21,22] and its segregation at the lamellipodium generates both extracellular and intracellular surface H^+ gradients from the rear end to the leading edge.[14−16] Incidentally, polarized segregation of raft and/or ganglioside microdomains does occur in migrating cells.[23−25] Migration along an exterior pH gradient has been observed for the amoeba *Amoeba proteus*[26] as well as osteoblastic cells.[27] Polarized epithelial cells also display NHE-dependent external pH gradients between the apical, lateral and basal sides.[17,18] Notably, differences in lipid rafts and ganglioside distribution between the apical and basal membranes have also been established for epithelial cells.[28] A highly polarized cell tissue for which a spectacular transverse pH gradient (2.5 units) occurs is the *Stratum corneum*,[29] i.e., the upper layer of the epidermis. Lipids extracted from the *Stratum corneum,* contain gangliosides[30] and exhibit a complex phase behavior as a function of pH.[31]

In the present study, we have investigated the effect of local lateral pH gradients on lipid domain dynamics by using localized acidic solution microinjection on electroformed giant unilamellar vesicles containing raft-mimicking Lo domains or phases with special emphasis on the role of the acidic ganglioside GM1.

MATERIALS AND METHODS

Reagents. Lipids were obtained as follows and used without further purification: egg yolk l-α-phosphatidylcholine (PC), egg yolk sphingomyelin (SM), cholesterol (Chol), and ovine brain GM1 were from Avanti; the fluorescent lipid analogue Texas Red DPPE (TR-PE) was from Invitrogen.

Giant Unilamellar Vesicles Preparation, Video Microscopy, and Microinjection. GUVs were made by the electroformation method on platinum electrodes in a temperature-controlled chamber with Hepes 0.5 mM, pH 7.4 as buffer, and viewed by phase contrast and fluorescence microscopy as described previously.[32,33] The setup used for microinjection was similar to earlier studies.[34−36] Micropipets with internal diameter 0.3 μm were used. The injection pressure was usually 25−30 hPa and the distance from the micropipet tip to the GUV surface was 30−60 μm. Image and movies were background-corrected with the "subtract background" routine and further processed with the "unsharp mask" filter of ImageJ.[37] For quantitative determination of the direction and kinetics of Lo and Ld phase polarization in GUV image series, the following ImageJ procedure was designed. A mask was manually constructed to crop the vesicle fluorescence from background, electrode, edge, and halo fluorescence. All images were thresholded for bright pixels and denoised using the " threshold " and " remove outliers " routines. The centroid (barycenter) of bright pixels was then determined for each image using the "moment calculator" plugin.[38] The procedure was repeated on the negativized image series for the centroids of dark pixels. The direction and modulus of the vector connecting both centroids was then computed for the image series.

Large Unilamellar Vesicle Preparation and Spectroscopic Fluorescence Measurements. Large unilamellar vesicles (LUV) were prepared using the extrusion method, in HEPES 5 mM, pH 7.4 EDTA 0.1 mM as described previouly.[34] The fluorescent probe LAURDAN was mixed with the lipids in the initial organic solution at a LAURDAN/lipid mole ratio of 1:200. Steady-state fluorescence emission spectra were recorded at 18 °C at a total lipid concentration of 0.2 mM as described.[34] For pH studies, the pH of the LUV samples was gradually lowered by adding aliquots of 10 or 100 mM HCl. Measurements were made after 5 min of equilibration under agitation. Excitation wavelength was 355 nm. Emission spectra from 370 to 600 nm were recorded twice, background subtracted and averaged. Emission intensities at 440 and 490 nm, I_{440} and I_{490}, respectively, were also specifically measured 10 times and averaged. Shifts in the LAURDAN emission spectrum were quantified by calculating the generalized polarization (GP) defined as $GP = (I_{440} - I_{490})/(I_{440} + I_{490})$.[39]

RESULTS

In order to study the effect of a lateral local pH gradient (see ref 40) on microdomain dynamics with emphasis upon the role of GM1, we mainly made use of heterogeneous Lo/Ld GUVs in which the Lo phase was generated by photosensitization. Fluorescence probes used for imaging Lo/Ld phase separation in GUVs also have a photosensitizing effect, thereby generating lipid species that promote Lo domain formation.[41,42] Recently, oxidized phosphatidylcholine has been identified as a product of such photosensitization and might be involved in Lo domain formation.[43] Other authors have suggested a role of oxidized sphingomyelin[41] or phospholipid polymerization.[42] While previous authors have appropriately pointed out the potential artifacts associated with such processes,[41,42] we have shown previously[33] that, under controlled conditions, this effect can be used as a tool. In the framework of the present study, the use of photoinduced Lo domain here has two advantages. First, photoinduction allows one to rapidly trigger uniformly distributed microdomain formation and to study associated dynamic processes on a faster time scale compared to other methods such as, e.g., temperature variation. Second, under specific experimental conditions, photoinduction produces either discrete Lo domains over a continuous Ld phase or to discrete Ld domains over a continuous Lo phases respectively above and below a specific temperature. Here, the effect of a local pH gradient on both phase morphologies was potentially interesting to study. We used the same type of lipid molar composition than that of our previous study, namely PC/SM/Chol/GM1 50:(30 − X):20:X, X being the molar proportion of GM1 varying from 0 to 10. The fluorescence probe TR-PE (0.25% molar) was used for domain imaging (it is excluded from the Lo phase) and also promoted the photosensitizing effect. Whenever possible, results obtained with photoinduced Lo domains were cross-checked with nonphotoinduced "preformed" Lo domains which result from spontaneous phase separation in the dark and therefore can be observed from the very beginning of illumination. Such preformed Lo domains occur in the 0−1% GM1 range below 19−22 °C.[33]

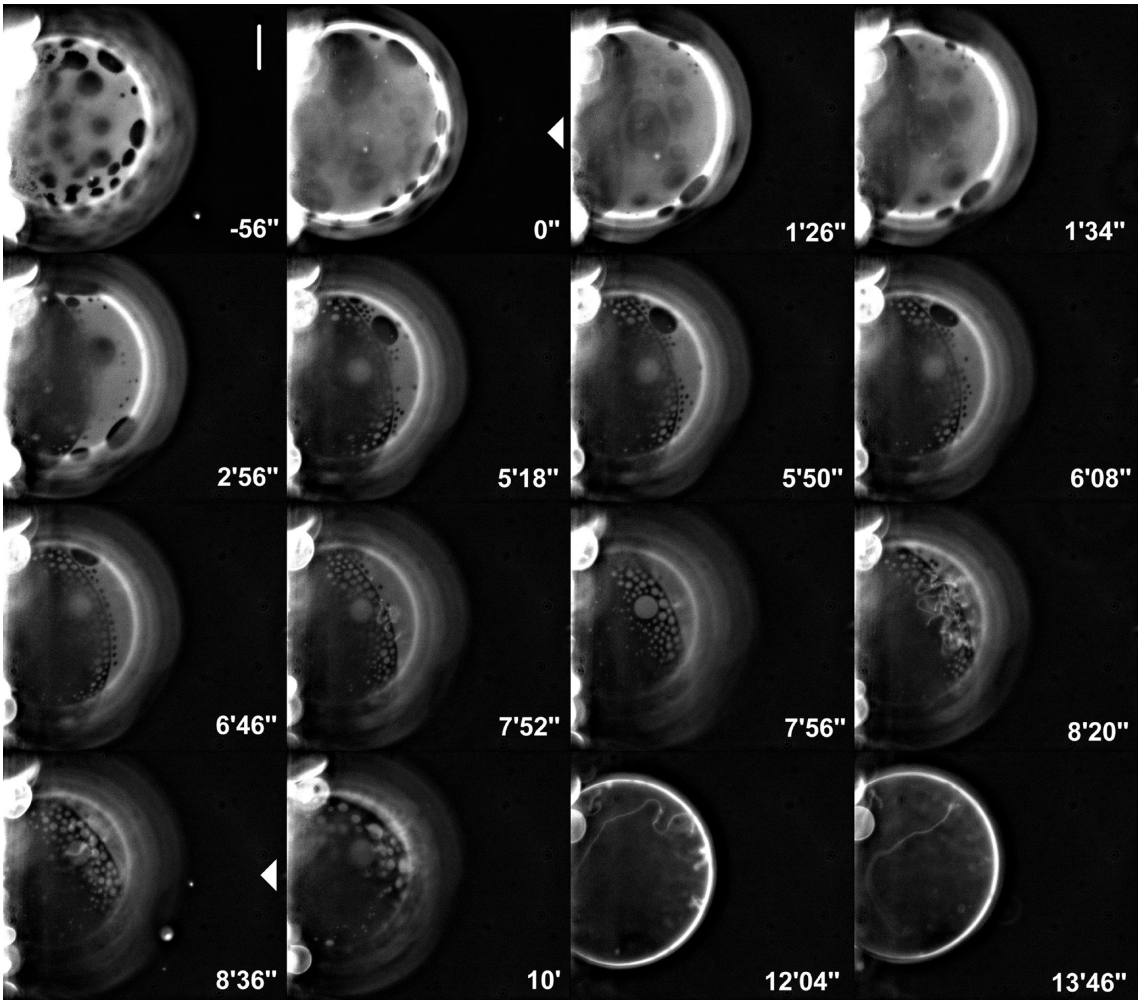

Figure 1. Fluorescence microscopy images showing the time-dependent development of lateral segregation, vesicle polarization, and Ld phase tubulation on photoinduced Lo/Ld phases after local external acidification by 100 mM HCl injection (25 hPa pressure) at 28 °C on a single GUV with molar composition PC/SM/CHOL/GM1 50:28:20:2 (mol/mol) labeled with TR-PE (0.25% mol). The times before or after the beginning of acidification are indicated for each image as negative or positive values respectively. White arrows indicate the position of the micropipet tip as well as the start and end of acid microinjection. Bar: 20 μm.

In order to establish a spatial lateral pH gradient at the GUV membrane surface, we used local delivery of an acid solution (usually 100 mM HCl at a 25−30 hPa pressure) in the vicinity of the vesicle using a micropipet.[34−36] This procedure generates a curvilinear pH gradient along the GUV outer surface, as directly visualized previously using a membrane-bound pH sensitive fluorescence probe.[36] Here the micropipet tip was positioned 40−60 μm from the vesicle membrane in order to create a lateral pH gradient involving a larger part of the membrane surface and the acid flux was imposed for several minutes. Estimation of the steady state pH at the membrane surface was made using a new theoretical treatment taking into account the microscopy imaging of the interior geometry of the micropipet to model the proton flux (Bitbol A. F., to be published) and, using 5 and 0.3 μm for the length and diameter of the outer tip, yielded values of 4.75 to 5.0 at the membrane surface facing the micropipet. Microinjection was started at a specific stage of photoinduced Lo phase separation corresponding to evenly distributed and relatively round-shaped microdomains of 5−10 μm size.

Effect of Local Acidification on Lipid Microdomain Dynamics in the Absence of GM1. As a control, before investigating the role of the acidic lipid GM1, we first studied the effect of establishing an external acid pH gradient in the vicinity of GUVs without GM1 (PC/SM/Chol 50:30:20, mol/mol). At 27 °C, with such composition, phosensitization gives rise to discrete Lo domains which diffuse within an Ld phase and progressively coalesce. We submitted such GUVs to continuous acid local microinjection. No detectable effect of the local pH gradient over the dynamic behavior of the photoinduced Lo domains was observed. Domains kept on diffusing randomly and progressively coalesced, eventually forming an Lo cap, insensitive to the presence of the pH gradient (Figure S1, Supporting Information). This was true with HCl concentrations ranging from 10 mM to 6 M. Below 22 °C, such composition gives rise to a spontaneous phase separation in the dark for the majority of vesicles, which from the beginning of fluorescence microscopy observation display large preformed Lo domains that rapidly coalesce into a cap. In the absence of GM1, the dynamics of these spontaneous preformed Lo domains was found to be also insensitive to formation of a pH gradient (not shown). As explained in our previous work, the occurrence of the preformed Lo domain precludes generation of photoinduced Lo domains. However, at

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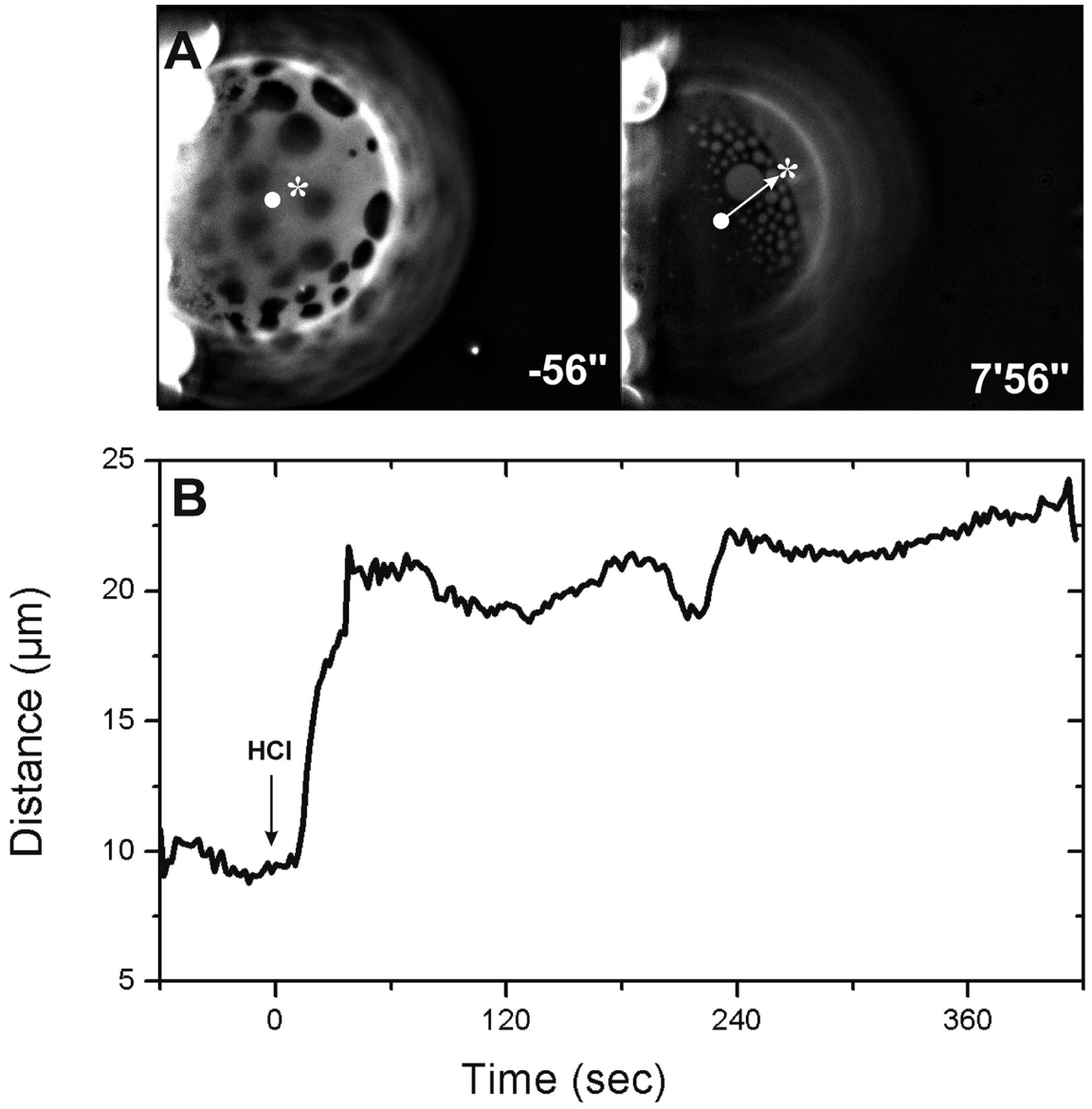

Figure 2. Quantification of lateral segregation and vesicle polarization on photoinduced Lo/Ld phases after local external acidification at 28 °C on a single GUV with molar composition PC/SM/CHOL/GM1 50:28:20:2 (mol/mol) labeled with TR-PE (0.25% mol), corresponding to the data of Figure 1 and movie S1 (Supporting Information), from the evolution of the centroids (barycenters) of bright and dark pixels. A. Position of the centroids for dark (white circle) and bright (white star) ca. 1 min before and ca. 8 min after local external acidification. B. Time evolution of the distance between both centroids after acid injection.

17 °C, a few vesicles (<5%) were found to be initially homogeneous and devoid of preformed Lo domains, presumably due to slight variations in composition inherent to the vesicle formation process.[31] Such vesicles did undergo formation of a photoinduced phase separation that produced discrete micrometric Ld domains over a continuous Lo phase. The dynamics of such Ld domains was also insensitive to establishment of a pH gradient (Figure S2, Supporting Information).

Effect of Local Acidification on Lipid Microdomain Dynamics in the Presence of GM1. A rather different behavior is observed when 2% GM1 is incorporated in the GUVs (i.e., PC/SM/Chol/GM1 50:28:20:2, mol/mol). At 28 °C, when continuous HCl microinjection is started on a GUV with a morphology initially consisting of discrete photoinduced micrometric Lo domains over a continuous Ld phase, there is a definite segregative movement of both phases in opposite directions with respect to the proton source. A clustering and coalescence of Lo domains toward the region of lower H^+ concentration, opposite to the side of the vesicle where acid is injected, is evident, leaving a cap of nearly pure Ld phase on the acid-injection side. This process ultimately give rise to a directionally "polarized" vesicle with an Lo pole located toward the higher pH side together with an Ld pole located toward the lower pH side, aligned in the axis of the proton source (Figure 1 and Movie S1, Supporting Information). Occasionally, very small Lo (respectively Ld) domain become trapped within the Ld (respectively Lo) cap during the segregation process and gather in a nearly regular fashion on both sides of the interface under the influence of the pH gradient. Although the very small Lo domains ultimately fuse with the Ld/Lo interface, the very small Ld domains remain trapped along the interface. Such phenomena are common in microemulsions[44] (see ref 45). Once such polarization of the vesicle into two continuous poles

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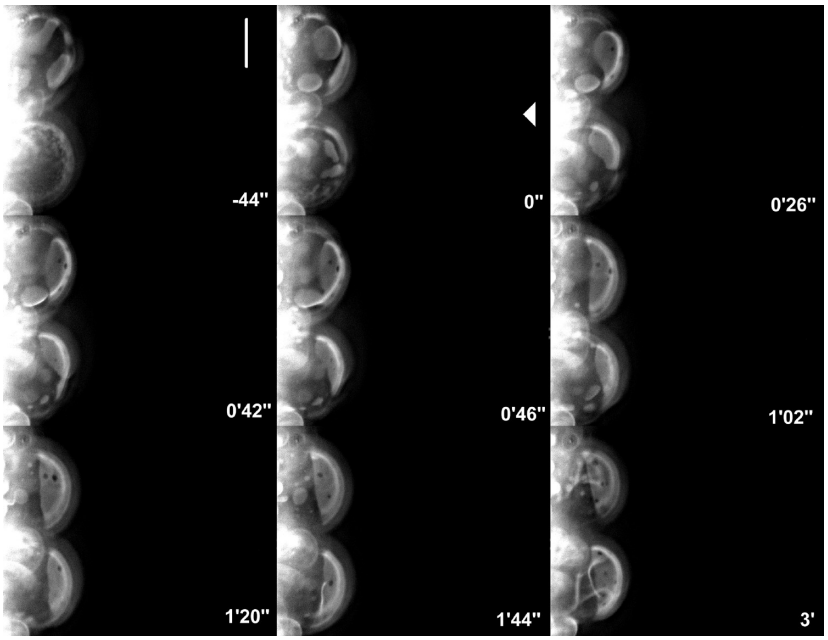

Figure 3. Fluorescence microscopy images showing the time-dependent development of lateral segregation and vesicle polarization on photoinduced Lo/Ld phases after local external acidification by 100 mM HCl injection (25 hPa pressure) at 20 °C on two adjacent GUVs with molar composition PC/SM/CHOL/GM1 50:28:20:2 (mol/mol) labeled with TR-PE (0.25% mol). The times before or after the beginning of acidification are indicated for each image as negative or positive values respectively. The white arrow indicates the position of the micropipet tip as well as the start of acid microinjection. Bar: 20 μm.

is established, a sequentially later process is the protrusion of inward tubes from the Ld phase lipids toward the vesicle interior. The whole polarization/tubulation process is only partially reversible. When acid injection is switched off, the vesicle remains bipolar but the Ld and Lo poles move away from their directionally polarized position with regard to the micropipet. On the other hand, tubulation from the Ld phase appears largely irreversible. Occasionally, we observed formation of very long tubes as shown in Figure 1.

In order to quantify the kinetics of lateral segregation and polarization of Lo/Ld phases after local external acidification shown in Figure 1, the spatial distribution of both phases was computed through their respective centroids (barycenters). As shown in Figure 2A, the centroids move apart with time along an axis that monitors the direction of polarization. The time evolution of the distance between both centroids is shown in Figure 2B. There is a ca. 1 min rapid phase followed by a slower phase that correspond respectively to the initial segregation of domains and their subsequent coalescence.

Similar experiments were performed at 20 °C with an initial photoinduced phase separation morphology consisting of discrete Ld domains over a continuous Lo phase, i.e., inverse from that found at 30 °C. After the start of acid injection, Ld domains segregate toward the acid injection side and fuse, ultimately forming an Ld cap while the Lo phase becomes concentrated toward the opposite side. Therefore, a polarized vesicle with an Lo pole located toward the higher pH side together with an Ld pole located toward the acid injection side, i.e., the lower pH side is obtained again, similarly to 30 °C. Tubes originating from the larger domains of the Ld phase and protruding toward the vesicle interior appear subsequently (Figure 3 and Movie S2, Supporting Information).

Hydrodynamic effects associated with continuous microinjection were found not to affect microdomain dynamics under our conditions (see ref 46).

Effect of GM1 Concentration on Local Acidification-Induced Lipid Microdomain Clustering. The data described above indicate that the ganglioside GM1 is specifically responsible for the observed domain segregation and tubulation effects. We therefore studied the effect of a local lateral pH gradient on photoinduced Lo phase dynamics at lower and higher GM1 concentrations. At 1% GM1 (i.e., PC/SM/Chol/GM1 50:29:20:1, mol/mol), the overall effect of the exterior pH gradient is similar although less pronounced. A less brutal segregation of Lo and Ld phases on opposite faces of the vesicles and a weaker tube triggering were usually observed. At 30 °C, there is a definite segregation of photoinduced Lo domains away from the more acid side of the vesicle leaving a remaining single Ld phase on this side (Figure 4 and Movie S3, Supporting Information). Tube formation is more limited and sometimes non observable, as in the vesicle of Figure 4. The reversibility of the phase poles orientation process is clearly visible in Figure 4. Although, during acid-induced polarization, the "rear" Lo phase pole becomes unobservable due to obscuring by the electrode and nearby small vesicles, when acid microinjection is switched off, it diffuses back to the microinjection side of the vesicle.

With 1% GM1, at 18 °C, as described previously[33] and as in the absence of GM1, a spontaneous phase separation occurs in the dark for the majority of vesicles. These display preformed Lo domains from the very beginning of illumination, that rapidly form a diffusing Lo cap. Continuous local acidification promotes the progressive movement of the preformed Lo cap away from the proton source and an opposite movement of the Ld cap toward the proton source. (Figure 5). Simultaneous formation of tubes from the Ld phase also occurs. Both the Lo and Ld caps remain stationary until interruption of the injection allow them to diffuse again. Thus the polarization effect of a local pH gradient found with photoinduced phases also occurs with lipid phases spontaneously preformed in the dark.

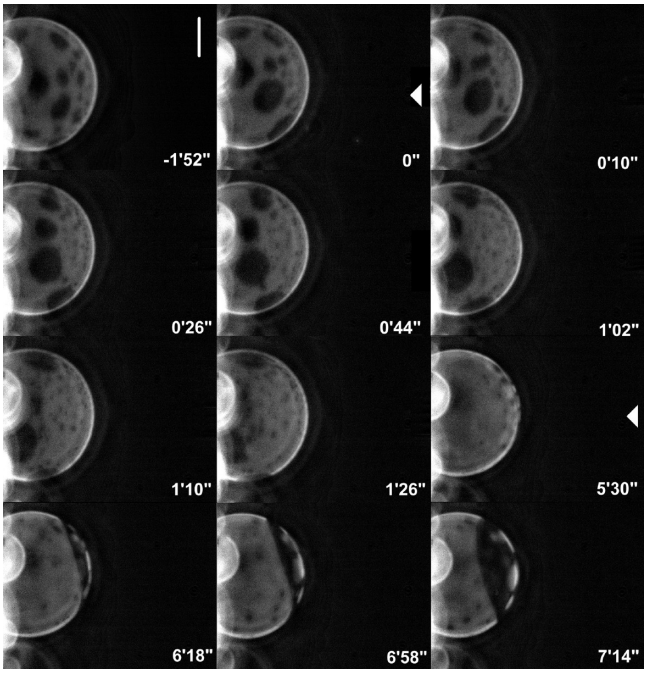

Figure 4. Fluorescence microscopy images showing the time-dependent development of lateral segregation and vesicle polarization on photoinduced Lo/Ld phases after local external acidification by 100 mM HCl injection (25 hPa pressure) at 30 °C on a single GUV with molar composition PC/SM/CHOL/GM1 50:29:20:1 (mol/mol) labeled with TR-PE (0.25% mol). The times before or after the beginning of acidification are indicated for each image as negative or positive values respectively. White arrows indicate the position of the micropipet tip as well as the start and end of acid microinjection.

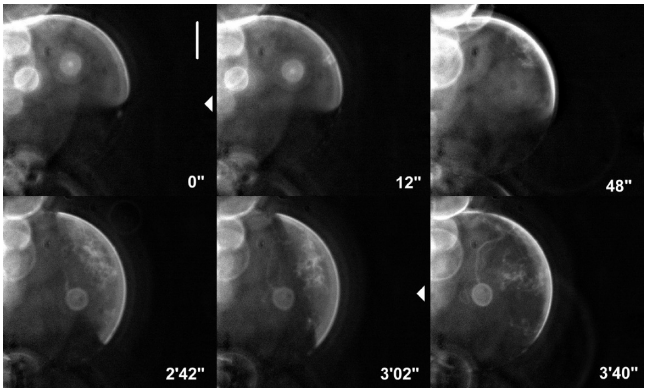

Figure 5. Fluorescence microscopy images showing the time-dependent development of domain reorientation, vesicle polarization and Ld phase tubulation on preformed Lo/Ld phases after local external acidification by 100 mM HCl injection (30 hpa pressure) at 18 °C on a single GUV with molar composition PC/SM/CHOL/GM1 50:29:20:1 (mol/mol) labeled with TR-PE (0.25% mol). The times before or after the beginning of acidification are indicated for each image as negative or positive values respectively. White arrows indicate the position of the micropipet tip as well as the start and end of acid microinjection. Bar: 20 μm.

Additionally, as observed at a similar temperature for 0% GM1 (see above), a few vesicles were devoid of preformed Lo domains and were found to undergo formation of a photoinduced phase separation that ultimately produced discrete Ld domains over a continuous Lo phase. Upon continuous local acidification, segregation of such Ld domains toward the acid side of the gradient is mostly less pronounced that at 2% GM1 but still occurs, giving rise to a visible Ld cap. A limited formation of inward tubes is occasionally observed (Figure 6 and Movie S4, Supporting Information).

At 10% GM1 (i.e., PC/SM/Chol/GM1 50:20:20:10, mol/mol) and 18 °C, tube formation from Ld phase domains is the dominant process, triggered immediately after the start of local acidification. In this case, tubes form even from small domains (Figure 7 and Movie S5, Supporting Information). Such fast and intensive tube formation obscures but also seems to partially hinder Ld domain segregation toward the acid side of the gradient although such segregation remains visible (Figure 7, time 4′52″). Segregated Ld domains rapidly diffuse away from the direction of microinjection when the latter is switched off.

In all, 11 GUVs preparations were examined, with GM1 content ranging from 0 to 10% mol, corresponding to 46 separate vesicle HCl microinjection experiments. The results are summarized in Table S1 (Supporting Information). Vesicle polarization or tubulation are never observed in the absence of GM1, whereas partial to complete polarization is almost always observed in the presence of GM1. The extent of tube formation increases with GM1 content. The proportion of vesicles undergoing complete or near complete polarization of Lo and Ld phases is maximal at 2% mol GM1. The overmentioned strong tubulation at higher GM1 content might partially hinder polarization. The time necessary to obtain maximum polarization of the vesicles was generally 3−5 min but occasionally shorter. Correlation between this duration and temperature or GM1 content is not univocal. It is likely dependent on other factors such as vesicle size and membrane tension.

As shown in our previous work,[33] at the chosen lipid compositions, photoinduced Lo domain formation is no more observed above a specific temperature which decreases with increasing GM1 content, so that GUVs remain in the Ld phase under these conditions. HCl microinjection experiments were also performed at such temperatures at 5 and 10% GM1 and showed extensive tubulation (not shown). Therefore, tubulation is a process intrinsic to the Ld phase, even under monophasic conditions.

Effect of Local Acidification on Lipid Microdomain Dynamics in the Presence of Asialo-GM1. In order to have a further insight into the chemical specificity of the role of GM1 in the induction of Lo/Ld domain vectorial segregation by a local pH gradient, we compared its effect with that of asialo-GM1 for which the branched sialic acid, carrying the acid carboxyl group is absent. The effect of asialo-GM1 itself on the Lo/Ld phase morphology behavior of heterogeneous GUVs is very similar to that of GM1 (unpublished). Therefore initial photoinduced phase morphologies similar to those with GM1 could be met. Spectacularly, at both 27 °C, for Lo domains over Ld phase (Figure 8A), and 17 °C, for Ld domains over Lo phase (Figure 8B), no effect of the pH gradient was found with GUVs containing asialo-GM1. Behavior after the start of HCl microinjection was identical to that of GUVs without ganglioside. This proves that the domain segregating effect of the pH gradient is specifically due to the presence of the full ganglioside GM1 with its protonable sialic acid moiety.

Effect of pH on Lo and Ld Phase Packing in the Presence or Absence of GM1 and Asialo-GM1 Measured by LAURDAN Fluorescence. We then investigated whether the GM1-dependent pH effect on microdomain segregation and tubulation in GUVs had their counterpart at the molecular

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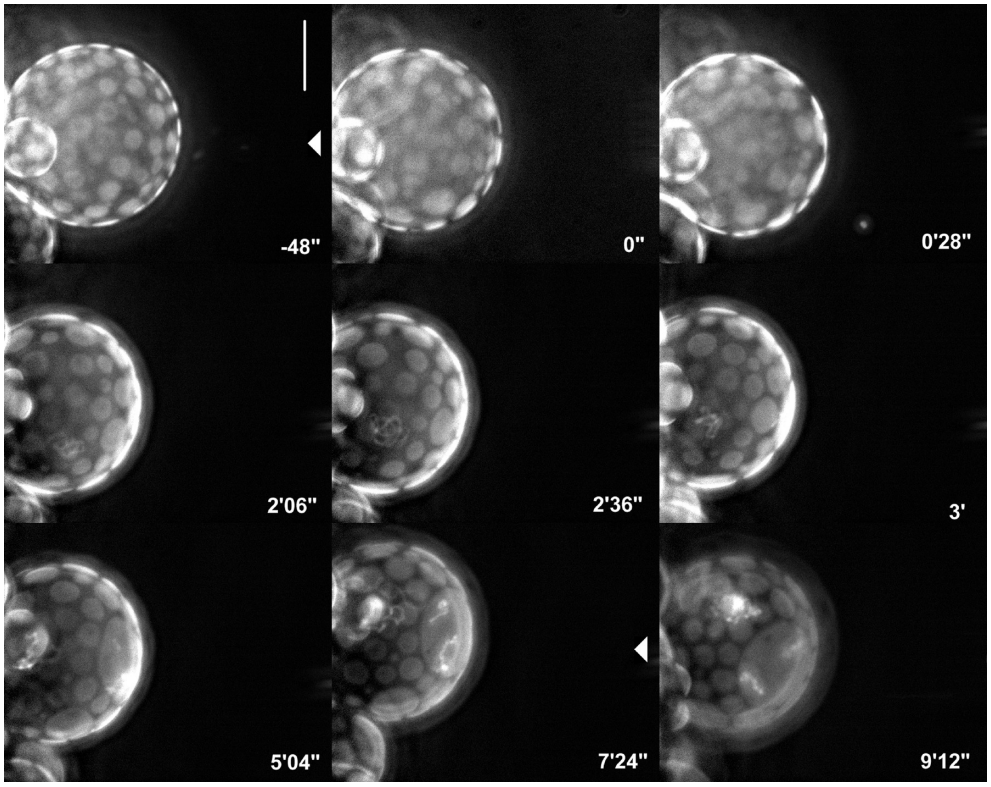

Figure 6. Fluorescence microscopy images showing the time-dependent development of lateral segregation, vesicle polarization and Ld phase tubulation on photoinduced Lo/Ld phases after local external acidification by 100 mM HCl injection (30 hPa pressure) at 18 °C on a single GUV with molar composition PC/SM/CHOL/GM1 50:29:20:1 (mol/mol) labeled with TR-PE (0.25% mol). The times before or after the beginning of acidification are indicated for each image as negative or positive values respectively. White arrows indicate the position of the micropipet tip as well as the start and end of acid microinjection. Bar: 20 μm.

level. We therefore studied the effect of pH on lipid packing properties using LUVs prepared by extrusion and LAURDAN as a fluorescence probe. LAURDAN fluorescence in lipid membranes undergoes a shift in its emission spectrum due to a packing-dependent environmental sensitivity of its excited state relaxation.[39] This is usually expressed as the general polarization GP which is a measure of lipid packing at the molecular level.

Figure 9A displays the effect of GM1 and pH on LAURDAN GP values for LUV lipid compositions typical of pure Lo (SM/Chol 50:50, mol/mol) and Ld (pure egg PC) phases. As expected, the overall GP values are much larger for Lo phase vesicles than for Ld phase vesicles. In the absence of GM1, for both Lo and Ld vesicles, no effect of pH is found above pH 3 and the GP is constant. As already discussed[34] the GP increase below pH 3 arises from counterion binding to the membrane rather than from phosphate group protonation. A first effect of GM1 is to increase the overall GP value at neutral pH mainly for Ld vesicles, a feature which can be related to the known ordering effect of GM1 which is even higher than that of SM.[47] Besides, the GP progressively becomes even higher with decreasing pH below pH 5.2. The most striking feature is that the latter increase is very important for Ld vesicle, whereas the effect for Lo vesicle is barely visible. The distinct effect of GM1 on both phases is further emphasized in Figure 9B where the difference in GP for Lo and Ld vesicles is plotted as a function of pH. Although in the absence of GM1 such difference is constant between pH 7 and 3, a large and continuous decrease of this difference is observed below pH 5.2 in the presence of GM1. Therefore, in the presence of GM1, the packing of the Ld phase becomes progressively closer to that of the Lo phase when the pH is decreased below 5.2.

Asialo-GM1 could not substitute to GM1 for this effect (Figure S3, Supporting Information). This indicates that the large increase in packing promoted by GM1 on Ld phase vesicles below pH 5.2 is indeed related to the presence of the protonable sialic acid moiety.

■ DISCUSSION

Our study is, to our knowledge, the first observation of segregative migration of lipid microdomains and directional lipid phase polarization under the influence of a naturally occurring chemical heterogeneity, namely a lateral pH gradient. Interestingly, Keatings et al. have previously shown phase segregation in GUVs in which a biphasic non biological polymer mixture was entrapped in order to mimick possible events in early cell evolution.[48,49] Our work also differs from previous investigations in which pH-induced phase separation of specific lipid molecular species in GUVs, starting from an initially homogeneous phase was triggered by a bulk pH variation.[50] In our work, a segregation of preexisting lipid microdomains was observed, a feature which requires a local pH gradient.

Most of the pH microinjection effects described above were obtained with GUVs in which Lo domains were generated by photosensitization. However qualitatively similar trends could be observed with preformed nonphotoinduced Lo domains, suggesting that the effect is not specific to photoinduced Lo domains. The use of photoinduced Lo domains allowed us to observe the very dynamics of these processes by triggering the

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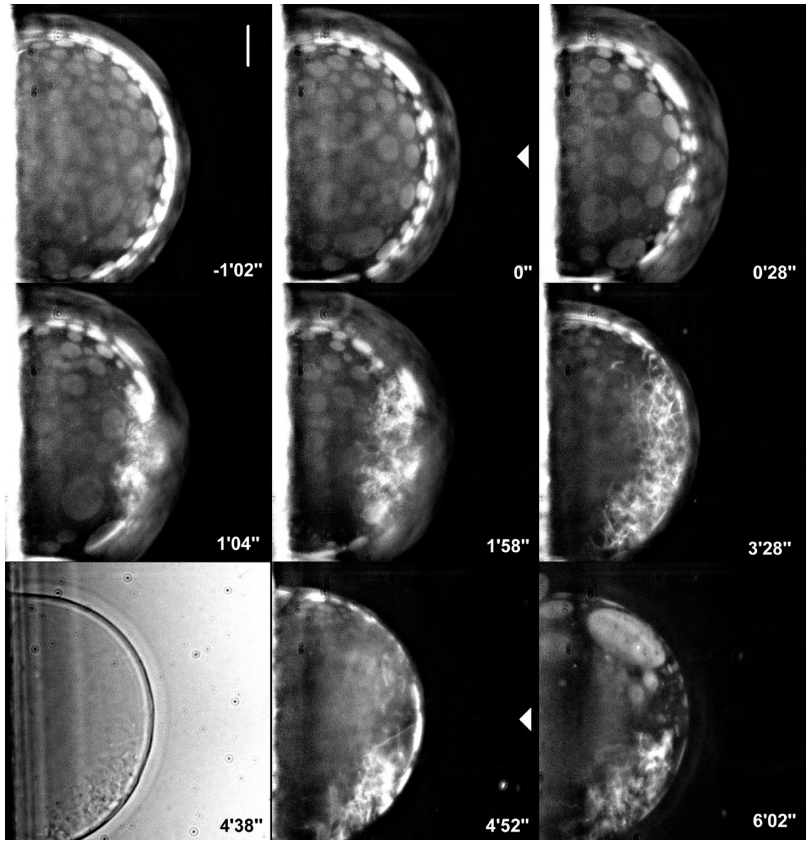

Figure 7. Fluorescence microscopy images showing the time-dependent development of lateral segregation and Ld phase tubulation on photoinduced Lo/Ld phases after local external acidification by 100 mM HCl injection (30 hPa pressure) at 18 °C on a single GUV with molar composition PC/SM/CHOL/GM1 50:20:20:10 (mol/mol) labeled with TR-PE (0.25% mol). A phase contrast image is shown at t = 4′38″ to emphasize tubulation. White arrows indicate the position of the micropipet tip as well as the start and end of acid microinjection. Bar: 20 μm.

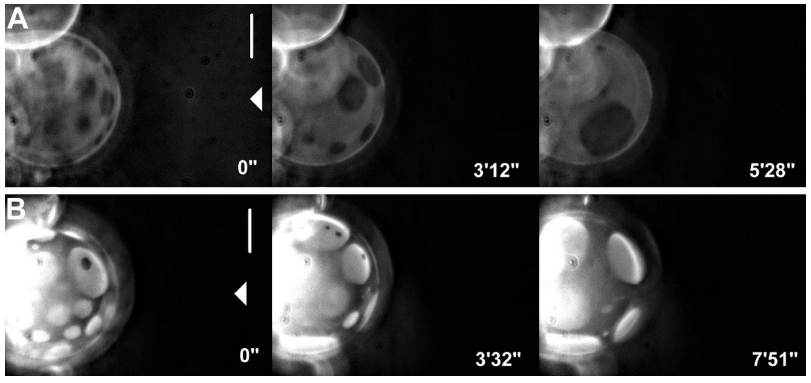

Figure 8. Fluorescence microscopy images showing the absence of lateral segregation and Ld phase tubulation on photoinduced Lo/Ld phases after local external acidification by 100 mM HCl injection (30 hPa pressure) at 27 °C (A) and 17 °C (B) on a single GUV with molar composition PC/SM/CHOL/asialo-GM1 50:28:20:2 (mol/mol) labeled with TR-PE (0.25% mol). The times before or after the beginning of acidification are indicated for each image as negative or positive values, respectively. White arrows indicate the position of the micropipet tip as well as the start of acid microinjection. Bar: 20 μm.

pH local gradient at the specific moment when domains reached a tailored size. It was clearly observed that the pH gradient promote a vectorial segregation of small domains eventually followed by their coalescence. This provides a closer mimick of actual cellular polarization processes. This confirms the usefulness of the photoinduction approach proposed in our previous paper.[33]

The present study shows that formation of an acid pH gradient in the vicinity of an Lo/Ld heterogeneous GUV containing the charged ganglioside GM1 mainly has two effects. One is segregation of Ld phase toward the acid side of the gradient and of the Lo phase toward the less acid side of the gradient. This occurs irrespective of whichever phase is continuous or consists of discrete domains and therefore of whichever phase is majoritary. The second is formation of inside-protruding membrane tubes at the acid side of the gradient, exclusively involving Ld phase lipids. The pH gradient-induced GM1-containing domain segregation is a quite novel observation. The LAURDAN fluorescence data are relevant for interpretation of the effect of lateral pH gradients

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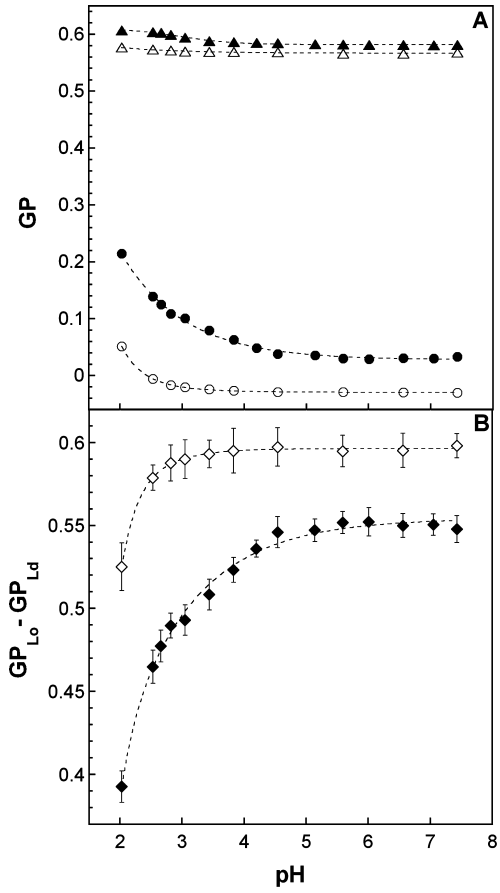

Figure 9. Effect of pH and GM1 on lipid packing in Lo and Ld phase LUVs as measured by LAURDAN fluorescence experiments at 18 °C. A. Evolution of GP with pH for LUVs composed of PC (open circles), PC:GM1 90:10 (mol/mol, closed circles), SM:Chol 50:50 (mol/mol, open triangles), SM:Chol:GM1 45:45:10 (mol/mol, closed triangles). B. Evolution of GP difference between Lo (SM/Chol) and Ld (PC) phase vesicles in the absence (open diamonds) or presence (closed diamonds) of GM1.

on biphasic GUVs. The difference of the effect of GM1 and acidic pH with regards to Lo and Ld phases packing can be interpreted as follows. Protonation of the sialic acid carboxyl abolishes the repulsion between GM1 molecules in the bilayer, promoting a general increase in lipid packing in the phase. However since the Lo phase is, as such, already nearly maximally packed, the further increase of packing that can be achieved by GM1 protonation is very limited. Oppositely, for the Ld phase which is intrinsically more loosely packed, protonation of incorporated GM1 can generate a much higher increase in lipid packing. Our proposal that the packing increase effect with acidic pH is indeed due to protonation of the GM1 sialic acid carboxyl is borne out by our observation that such effect is absent with asialo-GM1.

Although the pK_a of free sialic acid in solution is 2.6−2.7,[51] the apparent pK_a of GM1 sialic acid in LUVs or GUVs is likely to be shifted to higher values. Indeed, for acidic phospholipids in membranes, dissociation is accompanied by an increase of the negative surface charge density which increases the local submembranous proton concentration so that the apparent pK_a is anticooperatively increased.[52] Therefore it appears likely that the increase of membrane packing observed in LAURDAN experiments of GM1-containing GUVs below pH 5.2 is due to GM1 sialic acid protonation. Our calculations of the steady state pH at the membrane surface during GUV HCl microinjection experiments yield compatible values of 4.75 to 5. This reinforces the idea that the domain clustering/polarization processes observed here are indeed related to a low pH-induced packing increase effect.

Partitioning of GM1 between Lo and Ld phases is another relevant issue in the interpretation of the effect of lateral pH gradients on Lo/Ld biphasic GUVs in the light of the LAURDAN packing data on homogeneous Lo or Ld LUVs. It appears from three different AFM studies that, although GM1 favors the Lo phase, it can also partition in the Ld phase depending on its average concentration and on the specific amount of the other lipid components.[53−55] Therefore it is reasonable to assume that, in our GUV studies, GM1 is distributed between the two phases.

The local lateral pH gradient-induced segregation of Lo and Ld domains may be tentatively and qualitatively interpreted as follows. The lateral pH gradient imposed to the heterogeneous GUV may give rise to a spatial gradient of chemical potential for both types of domains/phases, which could act as an effective force for segregation. Two energy contributions might be considered. First, as shown by the LAURDAN fluorescence experiments on LUVs, low pH, which protonates GM1 molecules and abolishes their mutual repulsion, promotes a much higher condensing and ordering effect on the Ld phase than on the Lo phase. This may lead to a lower free energy of Ld lipid domains at the acid side of the gradient (due to decrease of electrostatic repulsion and, possibly, stronger interactions between lipid molecules) and therefore to diffusion and gathering of Ld domains/phase toward this region of lower free energy. Note, however, that higher lipid packing is not straightforwardly associated with lower free energy. Second, as extensively documented, a salient factor in the thermodynamics of Lo/Ld domain coexistence is the interface excess free energy (effective line tension) between both domain types. This appears to be mainly related to a difference in bilayer thickness.[56] As shown by the LAURDAN fluorescence experiments on LUVs, low pH increases the packing density of the Ld phase much more than that of the Lo phase. It therefore may lead to a selective increase of thickness of the Ld phase resulting in a decrease of the thickness mismatch between both phases. This may also contribute to the segregation and polarization of Lo and Ld domains.

The inward tubulation effect of low pH can be explained in the framework of our previous experiments.[34] Using GUVs containing cardiolipin, another negative and protonable phospholipid, we have found that local acidification promotes similar inward tubulation. This was interpreted as due to acidic lipid protonation yielding changes in both lipid local density and spontaneous curvature in the outer leaflet. Such modifications promote curvature instability more rapidly than dissipation by relative sliding of both monolayers can occur. A similar argument holds for GM1. Again the LAURDAN fluorescence experiments on LUVs can be invoked to explain why, in the present case, such tubulation occurs only for the Ld phase considering that the condensing effect of low pH is much higher than for the Lo phase. Here tubulation was found to be irreversible. Reversibility of pH-induced membrane tubulation is a complex issue which depends on numerous factors such as membrane tension, deformation modulus and diffusion of protons inside the nanotube and is currently investigated in our laboratory.

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CONCLUSION

Most cells polarize as a result of an heterogeneous inner or outer environment of their plasma membrane: anisotropic cytoskeleton binding, local concentrations of extracellular cross-linking ligands, gradients of chemoattractants, adhesion to other cells, or the extracellular matrix. Several studies suggest that juxtamembrane lateral pH gradients may contribute to such heterogeneous polarizing environment. Our study shows that GM1-containing Lo and Ld domains, which are mimicks of cellular lipid microdomains, do segregate under the influence of such pH gradients. To the extent that GUVs can be considered as simplified cell models, we therefore suggest that pH-driven lipid microdomain clustering might play a role in cellular membrane segregation or polarization processes in which local lateral pH gradients or pH heterogeneities are known to be important, such as migrating cells, epithelial cells, oligodendrocytes and the skin *Stratum corneum*.

ASSOCIATED CONTENT

Supporting Information

Additional images illustrating controlled microinjection experiments on heterogenous GUVs and additional fluorescence experiments on LUVs. Table summarizing the results of 46 separate vesicle HCl microinjection experiments. Movies showing the time-dependent development of photoinduced Lo/Ld phases lateral segregation, vesicle polarization, and Ld phase tubulation after local external acidification of GUVs. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(46) As a control, the intrinsic hydrodynamic effect of continuous liquid injection on microdomain dynamics was assessed by submitting identical heterogeneous vesicles to injection of iso-pH buffer. At micropipette tip distance of 10 μm from the vesicle membrane (i.e., closer than in the above experiments). Microinjection pressures up to 50 hPa did not perturb the intrinsic domain movements which were similar to those in the absence of microinjection. Pressures higher than 50 hPa and up to 200 hPa did induce accelerated domain movements and domain vortexing. However no domain clustering at one or another side of the vesicle was observed at all tested pressures.
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