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Title: Cholesterol-dependent phase separation in cell-derived giant plasma membrane vesicles**Authors:** Ilya Levental^{*}, Fitzroy J Byfield[†], Primit Chowdhury[‡], Feng Gai[‡], Tobias Baumgart[‡], Paul A Janmey[§]

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SHORT TITLE: Cholesterol effect on cell membrane domains

SYNOPSIS Cell-derived giant plasma membrane vesicles (GPMVs) enable investigation of lipid phase separation in a system with appropriate biological complexity under physiological conditions, and were used to investigate the cholesterol dependence of domain formation and stability. Cholesterol level is directly related to the abundance of the liquid ordered phase fraction, which is the majority phase in vesicles from untreated cells. Miscibility transition temperature depends on cholesterol and correlates strongly with the presence of detergent-insoluble membrane in cell lysates. Fluorescence correlation spectroscopy reveals two distinct diffusing populations in phase-separated cell membrane-derived vesicles whose diffusivities correspond well to diffusivities in both model systems and live cells. These results extend previous observations in purified lipid systems to the complex environment of the plasma membrane and provide insight into the effect of cholesterol on lipid phase separation and abundance.

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INTRODUCTION

Several lines of evidence including the identification [1] and characterization [2] of detergent-resistant membrane fractions, anomalous diffusion of membrane bound tracers [3], and nanoscale aggregation of fluorescent proteins and markers [4, 5], have been synthesized into the hypothesis of cholesterol- and sphingolipid-enriched membrane rafts in the plasma membrane [6]. The current conception of rafts consists of transient nanoscopic domains within the bulk membrane which contain a variety of specific proteins and lipids. These domains have been proposed as platforms for the organization and concentration of signaling components [2]. Model system experiments using mixtures of synthetic lipids in monolayers [7], supported bilayers [8], and giant vesicles [9, 10] have reproduced and extensively characterized liquid phase demixing in mixtures of cholesterol and various phospholipids [11]. A consistent result across all model systems is that inclusion of cholesterol in lipid mixtures can result in liquid-liquid phase separation into a liquid-ordered (L_o) and a liquid-disordered (L_d) phase [11, 12]. The L_o phase is characterized by conformational ordering resembling a crystalline/gel phase [13], but distinguished from it by a high degree of rotational and translation mobility characteristic of the L_d phase [14]. This L_o/L_d coexistence has been proposed to be the physicochemical basis for plasma membrane rafts, which have been postulated to be ordered phase domains.

Although model system experiments have successfully recapitulated cholesterol-dependent liquid phase coexistence, they have offered no conclusive evidence that L_d - L_o phase immiscibility is physiologically related to cell membrane rafts. This limitation is due in part to the fact that model systems cannot replicate the tremendous complexity of the plasma membrane, both in the heterogeneity of lipid species and the inclusion of membrane associated proteins that affect the thermodynamics of lipid-mediated demixing. However, recent experiments using giant plasma membrane vesicles (GPMVs), cell-derived liposomes that maintain the lipid [15] and protein [16] diversity of the plasma bilayer, have shown temperature dependent liquid-liquid phase separation, similar to that observed in model systems [17]. This phase separation was found to segregate known protein and lipid markers of lipid rafts, providing a link between L_d - L_o phase separation in model systems and the lipid raft hypothesis in cellular plasma membranes. Recent experiments have suggested that GPMVs exist near miscibility critical points, and therefore that the

behavior of these complex lipid and protein mixtures can be understood by universally applicable scaling laws [18].

Here, we extend these studies by using fluorescence microscopy and fluorescence correlation spectroscopy (FCS) to characterize liquid-liquid phase coexistence in GPMVs and find good agreement between observations in GPMVs and purified lipid model systems regarding the dependence of phase abundance and miscibility on cholesterol fraction. This agreement implies that phase behavior in simple lipid mixtures can be used to approximate domain formation in the complex environment of the plasma membrane. Additionally, we find strong correlation between the temperature dependent separation of a raft phase in GPMVs and the detergent resistance of cellular membranes, suggesting that these two phenomena are related and that both are related to the existence of membrane domains in the native plasma membrane.

EXPERIMENTAL

GPMVs were isolated after chemically inducing cell blebbing with 25 mM paraformaldehyde and 2 mM dithiothreitol in calcium-containing buffer (150mM NaCl, 10 mM HEPES, 2 mM CaCl₂, pH 7.4) for 1 hr at 37°C as previously described [17], and labeled with rhodamine 1-stearoyl 2-oleoyl phosphatidylethanolamine (rhoPE, Avanti) and/or naphthopyrene (nap, Sigma) by incubation at room temp for 15 mins with 2.5 µg mL⁻¹ rhoPE and/or 10 µg mL⁻¹ nap. A chamber was created by making a square of silicon sealant (Dow Corning) on a BSA-coated coverslip, into the middle of which 20 µL of labeled GPMV suspension was deposited, followed by sealing of the chamber with another coverslip. The fluorescence of the vesicles was visualized using an inverted microscope (Leica) equipped with appropriate filter sets. Temperature was controlled using a Peltier temperature control stage (TS-4, Physitemp) at 10°C, unless otherwise noted. Percent surface area covered by the L_d phase was quantified by calculating the surface area of the spherical cap (SA_{cap}) covered by the rhoPE-rich (bright) phase using the relation:

$$SA_{cap} = 2\pi * r_{vesicle} * h_{cap}$$

where h_{cap} is the height of the spherical cap and equivalent to:

$$h_{cap} = r_{vesicle} - \sqrt{r_{vesicle}^2 - r_{cap}^2} \quad \text{if } L_d \text{ is the minority phase and,} \quad h_{cap} = r_{vesicle} + \sqrt{r_{vesicle}^2 - r_{cap}^2} \quad \text{if}$$

L_d is the majority phase.

$r_{vesicle}$ and r_{cap} are the radii of the vesicle and rhoPE-rich cap, respectively, and were measured using ImageJ software.

FCS was performed with a confocal microscope on GPMVs as previously described for giant unilamellar vesicles [19, 20]. Detergent resistant membranes were isolated on a discontinuous sucrose gradient as previously described [21]. Purification and quantification of membrane lipids were performed in accordance with published protocols [22, 23]. Further experimental details are available in Supplementary Methods.

RESULTS AND DISCUSSION

GPMVs derived from NIH 3T3 fibroblasts were stained with rhoPE (disordered phase tracer), and observed by fluorescence microscopy to quantify the relative abundance of L_d and L_o phases (the L_o phase is also referred to as "raft phase" because it enriches for raft components [17]). The results represented in Fig. 1 show that more than 70% of the surface area of vesicles derived from untreated cells is comprised of the raft phase, suggesting a continuous ordered bilayer in which disordered domains exist as inclusions (imaging performed at 10°C unless otherwise noted, although phase abundances were temperature independent over a wide range (10°C-24°C; Supplementary Fig. S1)). This finding is inconsistent with the concept of liquid-ordered domains as isolated and scarce lipid rafts, instead suggesting the possibility of a plasma membrane existing as a majority liquid-ordered continuum interrupted by disordered domains. The idea of a percolating raft phase is consistent with recent measurements of diffusivity of raft and non-raft protein and lipid markers [24], electron-spin resonance in live cells [25], and single cell detergent extractions [26].

Detergent resistant membrane fractions which formed the original basis for the raft hypothesis are enriched in cholesterol, suggesting that lipid rafts are liquid-ordered membrane structures enriched in, and possibly dependent on, the presence of cholesterol. We modulated cholesterol levels in cells prior to GPMV isolation, and in GPMVs isolated from untreated cells, to determine whether cholesterol affects the abundance and coexistence of the two liquid phases. Depletion of cellular cholesterol by treatment with methyl-beta cyclodextrin (MBCD - 5 mM) decreased cholesterol mole fraction by more than 20% in the derived GPMVs and resulted in significant changes to the relative abundance of the phases, more than doubling the relative abundance of the disordered, non-raft phase from 28% to more than 65% of the surface area (Fig. 1). Inversely, loading cells with cholesterol by treatment with cholesterol-saturated MBCD (decreasing GPMV phospholipid:cholesterol from 1:1 to 1:0.8) lead to a near disappearance of the L_d phase, from 28% to 5% surface area, causing these GPMVs to appear nearly dark with very small bright disordered patches. Similar results were observed when GPMVs were cholesterol loaded after isolation from untreated cells.

Although MBCD depletion of plasma membrane cholesterol strongly affected the phase abundance of derived GPMVs, this technique was unable to deplete cholesterol below ~35 mol% (Fig. S4). This limitation was overcome by direct MBCD treatment of vesicles following their isolation from cells, which resulted in non-circular, jagged, and ribbon-like domains (Fig. 1) similar in morphology to gel phase domains observed in cholesterol-free GUVs where demixing was the consequence of acyl chain length differences between the component phospholipids [20].

These cholesterol modulation data are consistent with purified lipid experiments that have shown cholesterol-dependent formation of a liquid-ordered phase, and the abolition or reduction of that phase when cholesterol was depleted [10, 27]. The induction of a non-fluid gel phase by wholesale depletion of cholesterol is consistent with $L\alpha$ /so separation in GUVs absent of cholesterol [20] as well as measurements in cholesterol-depleted live cells [28] and observations of viral lipid extracts [29].

In addition to phase abundance, cholesterol mole fraction affects phase separation in numerous simplified lipid model systems [11], prompting the hypothesis that the same effect might be observed in the complex lipid and protein mixture of GPMVs. Cellular cholesterol levels were manipulated as above, and the temperature-dependent phase separation of GPMVs derived from those cells was measured. Loading cells with cholesterol (~20% decrease in [PL]/[Chol]) decreased the average miscibility transition temperature (T_{misc}) from 24 to 21°C, whereas depleting cellular cholesterol increased T_{misc} to 32°C. Cholesterol depletion also produced a significant fraction (15%) of microscopically phase separated vesicles at 37°C, suggesting the possibility of cholesterol-dependent phase separation in complex membranes at physiological conditions (Fig. 2). This observation corresponds well to phase separation induced by cholesterol depletion of live cells at physiological temperature [26]. Additionally, the cholesterol mole fraction dependence of T_{misc} (Fig. 2) closely resembles the same dependence measured in model liposomes [27]. This agreement is particularly striking since not only the trends, but also the absolute values of the transition temperatures are similar for three-component GUVs and the much more complex cell-derived vesicles considered here, suggesting that findings in purified lipid systems can be extended to multi-component, cell-derived protein-lipid mixtures.

The findings presented above show that phase abundance and miscibility as a function of cholesterol in GPMVs correspond well to the same properties observed in simple systems. On the opposite extreme of complexity from purified lipid models of membrane rafts are the detergent-resistant membrane fractions that suggest the existence of biochemically distinct membrane domains in whole-cell lysates. To determine the relationship between fluid phase coexistence in GPMVs and the presence of a low density membrane fraction in detergent-lysed cells, the temperature dependence of these two distinct membrane phenomena was investigated by also performing sucrose gradient fractionation of cell membranes at different temperatures. The temperature profile of microscopically-observable phase coexistence in GPMVs from untreated cells followed a relatively abrupt transition from entirely phase-separated to microscopically uniform vesicles between 20-25°C. The temperature-dependent abundance of the mass of cholesterol in detergent resistant membrane fractions yielded a very similar temperature profile, with the detergent resistant fractions making up 20-25% (wt/wt) of the total cholesterol below the phase transition temperature of the GPMVs, but <10% above (Fig. 3 and S2). The temperature-dependent presence of a detergent

resistant component was confirmed by quantifying the sucrose gradient distribution of a protein reported to partition to detergent-insoluble rafts. Below 20°C, >50% of fluorescently labeled cholera toxin B (Alexa-488 CTB) was recovered in low density fractions, whereas <15% was detergent resistant above this average GPMV phase separation temperature (Fig. S3).

Although the requirement for low temperature and detergent treatment has led to intense scrutiny regarding the physiological relevance of detergent-resistant membranes [30], we find a strong correlation between detergent resistance and GPMV phase separation. This result suggests that detergent insolubility and the existence of a separated ordered phase in complex mixtures are related, and that both are related to membrane rafts. These results confirm findings from detergent extractions of mixed lipid vesicles [9, 31] and GPMVs [32] and argue against the artifactual induction of liquid phase coexistence by detergent as the sole mechanism for raft formation. Although neither microscopically observable phase separation nor detergent resistance (without cyclodextrin treatment) was observed in our experiments at physiological temperature (37°C), the phase separation in GPMVs and detergent resistance of certain membrane fractions might occur due to the coalescence of underlying nanoscopic assemblies of raft lipids and proteins, recently proposed thermodynamically [18] and experimentally [33], that are neither microscopically observable nor detergent insoluble at 37°C.

One of the distinguishing characteristics of ordered versus disordered fluid phases in model lipid systems is a difference in translational and rotational diffusivity [13] and lipid and protein diffusivity are major determinants of the cellular distribution and corresponding functions of plasma membrane components. Lipid diffusivity was quantified in both phase-separated and uniform GPMVs by FCS on fluorescent tracer lipids incorporated into the vesicles. At a temperature at which GPMVs separate into two liquid phases (10°C), we observed two distinct populations of diffusion coefficients obtained from fits to autocorrelation data (Fig. 4). The histogram fitted to normal distributions yields average diffusivities of 1.8 and 5.6 $\mu\text{m}^2/\text{sec}$. The correlation data at 37°C suggest a single population of diffusion coefficients with a mean value roughly equivalent to that of the faster diffusing component at 10°C (Fig. 4b). These results agree well with previous measurements in both cells and model systems. The three-fold difference in lateral mobility between the two phases is almost exactly the same as was measured by fluorescence recovery in DMPC-cholesterol bilayers at physiological temperature [13]. The diffusion coefficients measured in L_o and L_d phases are very close to the diffusion coefficients measured here, strongly suggesting that the 1.8 $\mu\text{m}^2/\text{sec}$ component corresponds to the L_o (raft) phase of GPMVs while the faster component is likely the disordered phase. The diffusivity differences and magnitudes measured here correspond very well to those measured by FCS for L_d and L_o phase markers in raft-composition GUVs [19] as well as to small-scale diffusivities recently measured in live cells by optical tweezers [34], underlining the agreement not just in phase separation, but also in the properties of those phases, among live cells, GPMVs, and purified lipid systems.

The results presented here demonstrate that complex multi-component lipid and protein membranes can exhibit similar phase behavior as simple 3-component model membranes with respect to abundance, diffusivity, and phase separation. Our findings also suggest that this behavior is related to the detergent-resistance of biological membranes, thereby relating biochemical and biophysical descriptions of membrane rafts and suggesting an important role for GPMVs as an intermediate lipid raft model system combining the complexity of the biological membrane with the observable phase separation and experimental simplicity of purified lipid mixtures.

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FIGURE LEGENDS

FIGURE 1 - Fluorescence images and quantification of L_d (non-raft) phase fraction in GPMVs (stained with rhoPE to label the L_d phase) as a function of cholesterol modulation showing that cholesterol level relates inversely to the abundance of the L_d phase in GPMVs and that the L_o phase is the majority phase in vesicles from untreated cells. Ribbon-like gel phase domains are observed when cholesterol is entirely depleted by direct treatment of GPMVs. Error bars are SD from >35 vesicles per condition; scale bars are 5 μ m.

FIGURE 2 - Temperature-dependence of phase separation in GPMVs isolated from untreated cells (black), cholesterol-depleted cells (red circles), and cholesterol-loaded cells (blue squares). Pictures are superpositions of red and green images from epifluorescence micrographs of GPMVs prepared from untreated cells stained with rhoPE (L_d -red) and naphthopyrene (L_o -green) at 10°C (left - phase separated) and 37°C (right - homogeneous).

FIGURE 3 - Correlation between the temperature dependence of phase separation of GPMVs (red points; line is a sigmoidal fit) and the abundance of detergent resistant membranes (detergent solubilization performed at the indicated temperatures) as quantified by the percentage of cholesterol in the detergent resistant fractions (striped bars; error bars are standard deviations from three experiments). Low temperatures, which induce GPMVs phase separation, also induce Triton-resistant membrane fractions as quantified by the presence of cholesterol in low density fractions (1-3 for all temperatures except 10°C where DRMs were in fractions 1-6; see Fig. S2).

FIGURE 4 - Histograms of diffusion coefficients obtained by fluorescence correlation spectroscopy of rhoPE diffusing in (A) phase-separated vesicles at 10°C and (B) microscopically uniform vesicles at 37°C. Diffusion coefficients were calculated by fitting autocorrelation data to a 2-component 2D diffusion equation (for experimental details see Supplementary Methods). These data show a single diffusing population of tracers in uniform vesicles and two distinct populations in phase-separated vesicles (bold lines are Gaussian fits to all data and thin lines in (A) show the component Gaussians). Histograms are from >70 measurements on 7-9 vesicles per condition.

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Figure 1

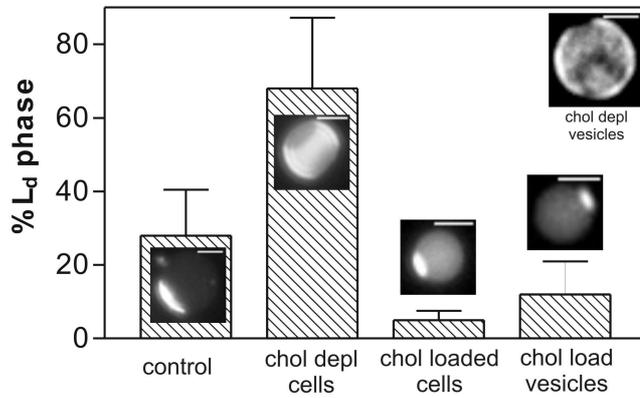
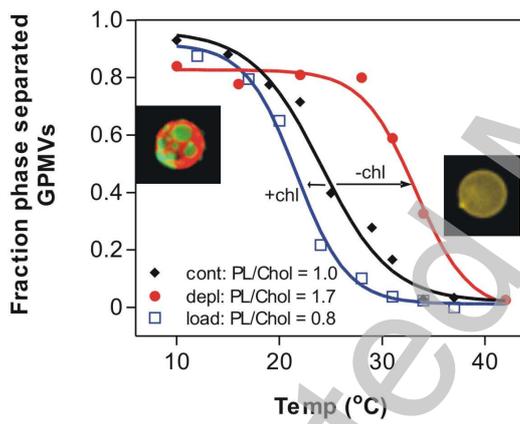


Figure 2



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Figure 3

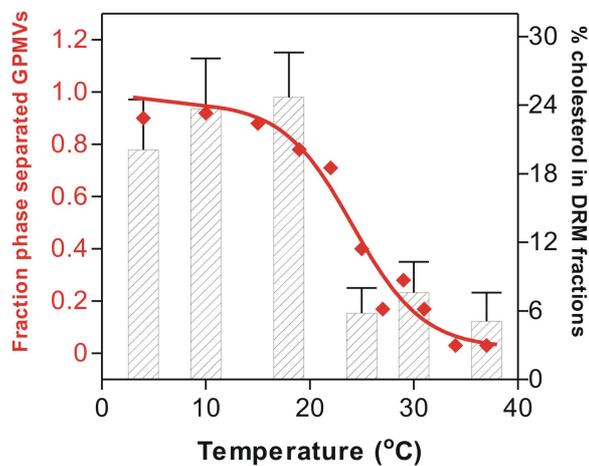
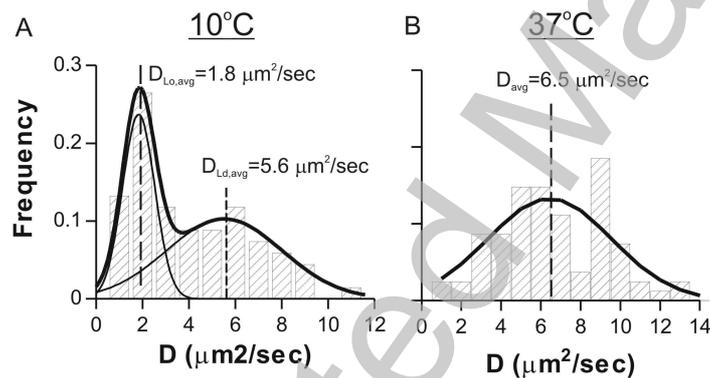


Figure 4



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