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Phase Composition Control in Microsphere-Supported Biomembrane Systems

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Abstract

The popularization of studies in membrane protein lipid phase coexistence has prompted the development of new techniques to construct and study biomimetic systems with cholesterol-rich lipid microdomains. Here, microsphere supported biomembranes with integrated α -helical peptides, referred to as proteolipobeads (PLBs), were used to model peptide/protein partitioning within DOPC/DPPC/cholesterol phase-separated membranes. Due to the appearance of compositional heterogeneity and impurities in the formation of model PLB assemblies, fluorescence-activated cell sorting (FACS) was used to characterize and sort PLB populations on the basis of disordered phase (L_d) content. In addition, spectral imaging was used to assess the partitioning of FITC-labeled α -helical peptide between fluorescently labeled L_d phase and unlabeled ordered phase (L_o) phase lipid microdomains. The apparent peptide partition coefficient, $K_{p,app}$, was measured to be 0.89 ± 0.06 , indicating a slight preference of the peptide for the L_o phase. A biomimetic motif of L_o phase concentration enhancement of biotinyl-peptide ligand display in proteolipobeads was also observed. Finally, peptide mobility was measured by FRAP separately in each lipid phase, yielding diffusivities of 0.036 ± 0.005 and $0.014 \pm 0.003 \mu m^2/s$ in the L_d and L_o phases, respectively.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information Available: A detailed description of the materials and methods.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

INTRODUCTION

The use of model membrane platforms to form liquid-liquid phase-separated biomembranes has gained significant interest as tools for the bottom-up study of membrane protein phase partitioning.^{1–6} Also of sizeable importance is the push toward increasingly biomimetic model membranes by incorporation of functional membrane proteins (MPs) and peptides, including functional ligand display.^{7–10} Membrane microdomains, encompassing the so-called lipid raft concept^{7, 11} are ordered nano- and microdomains in the plasma membrane that are typically sphingolipid- and cholesterol-rich and known to sequester a plethora of membrane proteins and alter their local concentration.^{8, 12} The formation of microdomains and the consequent compartmentalization of proteins within specific microenvironments are known to be compositionally dependent, affecting lipid and protein transport and chemical kinetics.^{2, 10}

Phase-separated membranes are typically studied in simplified platforms utilizing giant unilamellar vesicles (GUVs) and supported lipid bilayers (SLBs).^{7, 13, 14} GUVs are single-bilayer lipid vesicles with diameters between 1 and 100 μm , most usually formed by hydrating a dry lipid film placed on AC electrodes operating at low voltages.^{10, 15–19} SLBs, on the other hand, are planar membranes that self-assemble through the fusion of small unilamellar vesicles (SUVs) adsorbing to a hydrophilic flat surface, typically mica, quartz, or silica. Upon collision with the surface, SUVs deform, rupture, and spread as a single lipid bilayer separated from the solid support by a ~ 1 nm water layer.^{20, 21, 22, 23} Both platforms offer control of bulk lipid composition, simple preparation, integration with MPs and characterization by microscopy and spectroscopy. However, the solid substrate offers the advantage of increased mechanical stability of biomembranes due to electrostatic, steric, hydration, and weak van der Waals interactions between the lipid headgroups, water, and the underlying surface^{20, 23–25, 26} If liposomes are fused to solid microspheres, forming constructs known as proteolipobeads (PLBs), these cell-like assemblies can be analyzed by flow cytometry and also purified using fluorescence-activated cell sorting (FACS).

Considering the significance of interphase partitioning of lipid and membrane proteins, compositional control of model membranes emerges as an increasingly important tool. It has been shown that L_o/L_d microdomain systems share many attributes of the nanoscopic raft domains, and these synthetic membranes are the most well accepted model for such domains.⁵ Due to attributes such as bilayer thicknesses, NMR and EPR order parameter

dependence,^{27, 28} and temperature dependences, these systems are considered to be a good working model for relating raft nanodomains and MP phase partitioning. So far, the L_o/L_d partitioning of >10 MPs have been studied as a model for function in cellular raft nanodomains.^{3, 29–31} Studies of isolated plasma membranes have confirmed that L_o/L_d liquid–liquid phase coexistence is accessible in native-like membranes and the behavior evidenced is consistent with many aspects of the raft hypothesis.^{32–34}

Though the composition of both GUVs and SLBs are specified in the bulk sample preparation, it has been noted that resulting structures are compositionally heterogeneous and that equilibrium may be reached at long time scales.^{17, 35, 36} Phase composition-based sorting of individual PLB assemblies would therefore be an ideal way of both controlling and probing membrane protein microenvironment in PLB-based assays. Alternatively, this method could provide a route to isolate assemblies for biomimetic ligand display. A precedent has been established for both the formation of proteolipobeads with phase-separated supported biomembranes and membrane characterization by flow cytometry.^{37–39} However, these systems have not been utilized for composition-based sorting and protein phase partitioning.

Here, we present a new method to study phase-separated biomembrane systems that features the PLB platform for comprehensive population analysis and sorting based on phase composition. Heterogeneous PLBs with coexisting liquid-disordered (L_d) and liquid-ordered (L_o) domains were formed from α -helical-peptide ($K_3A_4L_2A_7L_2A_3K$ -Lys(FITC)-OH)/DOPC/DPPC/cholesterol lipid mixtures, characterized by confocal microscopy and flow cytometry, and then compositionally-sorted based on L_d phase content via FACS. In tandem, a new method to measure MP phase partitioning in the coexisting phases is also introduced, utilizing spectral imaging to quantify interphase behavior of our α -helical peptide that serves as a MP analogue for phase partitioning and mobile ligand display.

EXPERIMENTAL DETAILS

Materials

1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), cholesterol, and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl) (DOPE-cap-biotin) were obtained from Avanti Polar Lipids (Alabaster, AL). $K_3A_4L_2A_7L_2A_3K$ -Fmoc-Lys(Dde)-OH (α -helical peptide) was custom synthesized by Biobasic Inc. (Markham, Ontario, Canada). Fluorescein isothiocyanate (FITC), NHS-PEG₄-biotin, chloroform, N,N-dimethyl formamide (DMF), and methyl tert-butyl ether (MtBE) were obtained from ThermoFisher Scientific Corp. (Chicago, IL). Trifluoro-acetic acid (TFA, 95%), hydrazine, N,N-Diisopropylethylamine (DIEA), and piperidine were purchased from Sigma Aldrich (St. Louis, MO). Silica microspheres of 4.9 μ m nominal size were purchased from Bangs Laboratories (West Bend, IN). QDot® 705 Streptavidin Conjugate (QD705) and 1,1'-Didodecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) were obtained from Life Technologies, Inc. (Carlsbad, CA).

Proteolipobead Preparation

Prior to preparing lipid mixtures, α -helical peptide was received resin-immobilized for site-specific FITC labeling. The resin was first pre-swelled in DMF for one hour before incubating with 2% hydrazine in DMF for two-5 minute periods followed by washing. FITC was then conjugated to the deprotected ϵ -amine group of the N-terminal lysine by incubating overnight in a 25 mg/ml FITC solution with 5% DIEA. Following copious washing of excess FITC with DMF, the peptide N-terminus was then Fmoc-deprotected by incubation 20% piperidine for one hour. Remaining t-Boc protecting groups were removed and the peptide was simultaneously cleaved from the resin by a one-hour incubation with 95% TFA. The peptide was precipitated in MtBE, isolated by centrifugation and lyophilized.

Lipid/peptide mixtures containing 1:1 DOPC/DPPC, 30% cholesterol, 4% peptide-FITC were used with a L_d phase labeling scheme of 0.5% DOPE-cap-biotin³⁵ (all percents by mole). The mixture was prepared in chloroform, dried under vacuum overnight and hydrated at 2 mg/ml in PBS buffer forming a multilamellar vesicle (MLV) suspension. The MLV suspension underwent five freeze-thaw cycles in liquid nitrogen to allow even membrane labeling densities, followed by probe sonication in a 50 °C water bath using a BioLogics, Inc. 150 V/T Ultrasonic Homogenizer for 15 minutes, forming small unilamellar vesicle liposomes (SUVs). Aggregates and ultrasound probe remnants were removed by centrifugation. Silica microspheres suspended at 10 mg/ml in PBS were heated in the same water bath, pipetted drop-wise into the SUV suspension, briefly vortexed and then gently mixed. After a 30 minute incubation period, the resulting PLBs were then washed by 3 rounds of microcentrifugation-settling/rinsing in fresh PBS buffer. The liposome sonication and PLB fusion steps were conducted at 50°C, well above the reported liquid-liquid miscibility transition temperature for this mixture ($T_{\text{miscibility}} = \sim 29^\circ\text{C}$)⁴⁰ and higher than the highest lipid T_m in the sample ($T_m = 41^\circ\text{C}$) to allow for the greatest uniformity in composition possible when forming the PLBs. However, since we have incorporated our α -helical MP analog we limited the temperature to as close to physiological as possible. In other related studies, the (protein-free) samples were repeatedly incubated up to 80°C to prepare the supported biomembranes, most likely too extreme for studies of MPs due to the possibility of unfolding.³⁹ For reference, the peptide-free lipid mixture containing 1:1 DOPC/DPPC and 30% cholesterol is reported to give an L_o fraction of $\sim 50\%$ at 25°C in estimates based on tielines formed using GUVs with NMR and fluorescence imaging in tandem.⁴¹

PLBs were L_d phase-labeled by adding QD705 Streptavidin to form complexes with DOPE-cap-biotin lipids present within the supported membranes. Stock QD705-streptavidin was diluted 1:100 (0.5 $\mu\text{g}/\text{ml}$) in PBS. PLBs were then pipetted into the QD705-streptavidin suspension and incubated with mixing for 2 hours, followed by rinsing with PBS. In some cases the $K_3A_4L_2A_7L_2A_3K$ -Lys(FITC) peptides present within PLB membranes were then biotinylated by incubation with 1.3 mg/ml NHS-PEG₄-biotin in PBS for 1 hour, followed by rinsing. The biotinyl- $K_3A_4L_2A_7L_2A_3K$ -Lys(FITC)-containing PLBs were complexed with QD705-streptavidin at 0.5 $\mu\text{g}/\text{ml}$ in PBS for 1.5 hours, followed by rinsing and then subsequently imaged within 12–24 hours.

Imaging

Confocal microscopy was used to characterize the PLB constructs. A Zeiss LSM 710 confocal microscope was used to create 3D reconstructions of all samples and to measure apparent partitioning coefficient, $K_{p,app}$, via spectral imaging at the microsphere equator ($T = 25^{\circ}\text{C}$). A Leica SP8 confocal microscope set to 37°C was used for FRAP diffusivity analysis. With each instrument, a 63X/1.4 NA oil-immersion objective, a pinhole size of 1.0 airy unit, bidirectional scanning and two-line averaging were used. For Z-stack and FRAP experiments, FITC was excited using a 488 nm Argon laser and detected between 495 and 635 nm; QD705 was excited using 405 nm laser and detected between 650 and 780 nm. For spectral imaging, both the 405 nm and 488 nm lasers were active while emission was detected between 495 and 725 nm in increments of 5 nm. Z-stack experiments featured 1024×1024 pixel format with a voxel size of $0.057 \times 0.057 \times 0.5 \mu\text{m}$; spectral imaging, 1024×1024 with pixel dimensions of $0.033 \times 0.033 \mu\text{m}$; FRAP, 512×512 with $0.048 \times 0.048 \mu\text{m/pixel}$, 0.792 second time interval and bleaching on the third frame.

Flow Cytometry and PLB Sorting

Flow cytometry/fluorescence activated cell sorting (FACS) was used to characterize the distribution of fluorescent signal emanating from phase-separated PLBs on a per-microsphere basis and to isolate separate populations of biomembranes displaying discrete ranges of L_d domain coverage. A BD FACSARIA II Cell Sorter was used to accomplish the FACS. Peptide-FITC was excited using a 488 nm laser and detected with a 530/30 nm bandpass filter. DiI (L_d phase) was excited using a 488 nm laser and detected with a 576/26 bandpass filter. QD705 (L_d phase) was excited using a 633 nm laser and detected with a 730/45 nm bandpass filter. Single PLBs were isolated by gating the thickest band observed at the bottom range of forward scatter, the signature of single dispersed PLBs. From there, low, medium, or high L_d -phase populations were sorted based on gating relative ranges of fluorescence detected (arbitrary units) in either the DiI or QD705 (L_d phase) channels. An $85 \mu\text{m}$ nozzle sorting tip was used in High Sort mode and drop-drive frequency of 90 KHz to achieve analysis rates of $\sim 30,000$ per second. The conditions were arrived at based on consideration of three factors 1) starting PLB sample size limitations, 2) minimization of the dilution and PLB instability during sorting and 3) the achievable sorting yield per minute of intact PLBs. Pre-sorted and sorted populations were then imaged by confocal microscopy.

To quantify microsphere coverage of L_d phase lipids, confocal stacks were processed in imageJ one PLB at a time by doing the following: (1) Z-projection of QD705 or DiI signal from a single sphere into two hemispheres separated at the equator, (2) thresholding of each hemisphere's signal to differentiate L_d and L_o phases, and (3) ratioing of the total hemisphere L_d phase fluorescence (raw integrated density) to that of a PLB hemisphere standard with 100% coverage (giving a number between 0 and 0.5), and combining the values from each hemisphere. For DiI samples, this process was performed for $N = 50$ PLBs in each of three sort groups (Low-, Mid-, and High-DiI) as well as the Pre-Sort group. For the QD705 sample, this was done for $N = 34$ PLBs from the Pre-Sort group and $N = 28$ for the High- L_d and Low- L_d sorted groups.

RESULTS AND DISCUSSION

Phase-separated PLB Formation

Phase-separated proteolipobead (PLB) assemblies were made by fusing SUV liposomes containing DOPC, DPPC, cholesterol, and K₃A₄L₂A₇L₂A₃K-Lys(FITC) to 5 μ m silica microspheres. Figure 1 is a schematic of the three labeling schemes used in this investigation. In figure 1, labeling scheme I, both the L_o and L_d phases contain biotinyl-K₃A₄L₂A₇L₂A₃K-Lys(FITC) bound to streptavidin-Qd 705 and the L_d phase also contains the L_d-specific C-12 (Didodecyl) DiI lipid tracer. In labeling scheme II the K₃A₄L₂A₇L₂A₃K-Lys(FITC) α -helical peptide partitioned in the L_o and L_d phases is labeled by FITC and the L_d phase is labeled by streptavidin-QD705 bound to DOPE-cap-biotin. Phase separation is displayed by non-uniform membrane labeling of QD705-Streptavidin bound to DOPE-cap-biotin lipids known to partition to the liquid-disordered (L_d) phase enriched by DOPC.³⁵ In labeling scheme III, K₃A₄L₂A₇L₂A₃K-Lys(FITC) is partitioned into the L_o and L_d phases and the L_d phase is preferentially stained by the C-12 DiI lipid tracer.

Figure 2 contains CLSM 3D reconstructions of K₃A₄L₂A₇L₂A₃K₂-FITC α -helical peptide display and phase partitioning in 1:1 DOPC:DPPC with 30% cholesterol and 4% peptide-FITC. The image in panel A is a zoomed out 2-channel 3D CLSM reconstruction, displaying the phase heterogeneity and PLB aggregation/defects that result from the construction protocol. As indicated in labeling scheme I of figure 1, L_d phase is labeled by C12-DiI lipid reporter (red). The biotinyl-K₃A₄L₂A₇L₂A₃K₂-FITC α -helical peptide partitioned in the L_d and L_o phases is labeled by bound QD705-streptavidin (green (false color)). PLBs with all levels of L_d content are clearly visible, including those with nearly all L_o (completely green) and all L_d phase present (completely red). In some PLBs, indicated by white arrows, the L_d phase appears to be bulging from the surface as has been evidenced in 1:1

DOPC:DPPC/30% cholesterol GUVs brought down to below the liquid-liquid phase miscibility temperature ($T_{\text{miscibility}} = \sim 29^\circ\text{C}$). As these images were taken at 25°C , our findings are consistent with the earlier GUV L_d phase domain bulging phenomena reported by Veatch et al.⁴⁰ and Baumgart et al.⁴² This image is from a small fraction of the $\sim 10^6$ PLBs obtained from 1 mg of microspheres, thus this sample would be an appropriate starting material for further purification using FACS. Panel B, top graph, is a histogram of the raw DiI (L_d) channel integrated density (RID: in arbitrary units) per PLB obtained from the CLSM indicating the heterogeneity of L_d phase in a very broad distribution that is not centered at the average value ($\text{RID}_{\text{DiI,avg}} = 23.2 \pm 17.3 (\times 10^3) \text{ A.U.}$). This histogram reflects the differences in total L_d phase content per PLB mixed with the heterogeneity of loading of the DiI per unit area of the L_d phase. For comparison, the bottom graph of panel B is a histogram of the StrepAv-Qd705:Biotinyl-K₃A₄L₂A₇L₂A₃K-Lys(FITC) (L_d + L_o) channel integrated density per PLB (in arbitrary units) indicating the relative homogeneity of the α -helical peptide loading into the PLBs, as indicated by the symmetric distribution ($\text{RID}_{\text{Peptide,avg}} = 24.6 \pm 4.1 (\times 10^3) \text{ A.U.}$). Although this data was taken from a different channel, the width of the distribution is less than 4-fold narrower in the latter case, indicating consistent total α -helical peptide loading per PLB, however, the phase partitioning is not yet apparent in this distribution or the imagery.

Figure 2, image C is a zoomed in 2-channel 3D CLSM reconstruction, also displaying of the phase heterogeneity and PLB aggregation/defects. As indicated in labeling scheme II of figure 1, the L_d phase is labeled by DOPE-cap-biotin bound to QD705-streptavidin (red). The $K_3A_4L_2A_7L_2A_3K_2$ -FITC α -helical peptide in the L_d and L_o phases is labeled by intrinsic FITC (green). Heterogeneous distribution of L_d phase lipids is clear, along with membrane defects and lipid/peptide aggregates. Regions absent of or minimal intensity in QD705 signal were attributed to liquid-ordered phase (L_o) lipid coverage enriched in DPPC and cholesterol. As in image A, the constructs display a wide of range of microsphere surface coverage for the L_d domain. From these initial 3D images in A and C, peptide-FITC appears to distribute into both lipid phases without partitioning into either phase. In addition, impurities and constructs containing defects such as microsphere multiples/aggregates, interfacial peptide or quantum dot aggregates, or incomplete microsphere coverage were observed in minor fractions. It was also noticed during analysis that a distribution in QD705 intensity exists that is independent of L_d domain coverage. This indicates heterogeneous loading of DOPE-cap-biotin per unit area L_d phase, and thus freeze-thaw cycling was insufficient in homogenizing the biotinylated lipid throughout disordered phases in MLV suspensions prior to sonication. From the standpoint of the CLSM findings, it would be clearly beneficial to further purify these samples using FACS to eliminate impurities and defective PLBs or to isolate PLB subsets with tailored L_o phase compositions.

These CLSM results vary markedly from experiments conducted on peptide-free 1:1 DOPC:DPPC/30% cholesterol GUVs,⁴⁰ most likely due to the perturbing presence of both the peptide and silica substrate, coupled to the high degree of heterogeneity of phase composition. The smaller L_d domains do not become rounded and remain irregular edges as in the case of some GUVs, even when incubated for extended period at $T > T_{\text{miscibility}}$. However, other GUV systems imaged utilizing DOPC:DPPC/cholesterol gave similar jagged L_o/L_d phase boundaries.⁴³ Moreover, there appears to be some degree of domain ripening in the PLBs, as the L_d domains are large and continuous, with very few small isolated domains. Due to the high heterogeneity of PLB compositions resulting from SUV formation and liposome-microsphere fusion events (both above $T_{\text{miscibility}}$), as contrasted to specified values in the dried lipid/peptide film starting materials, each PLB biomembrane construct can be seen as a single phase-separation experiment of discrete composition within the $L_o + L_d$ coexistence region. Some minor fraction of the PLBs contain compositions outside the boundaries of the two-phase coexistence (all red or all green in Figure 2A: ~2 out of 50 PLBs). This type of intact lipid bilayer assembly heterogeneity of composition has been evidenced in many systems, including proteoliposomes and GUVs, most acutely in studies involving natural lipid systems with complex cell- or organelle-like compositions. A pertinent example is the work of Bezlyepkina et al., showing the highly complex size and phase heterogeneity of GUVs produced by electroformation, from images that contain a number of GUVs of various sizes attached to the electroformation wire.⁴⁴ Their measurements of area of L_d phase fraction ranged from 0.5 – 0.9 in electroformed GUVs. This heterogeneity and uncertainty in the lipid composition hampers the interpretation of MP studies in bulk proteoliposomes and limits sample sizes in GUV studies, as assays would require larger numbers of intact bilayer constructs of similar lipid microenvironment. This unmixing phenomena has been overcome in only limited ways, including electrofusion

of single composition controlled-GUVs,⁴⁴ allowing for precise control at only the single assembly level and, more recently, by using carefully selected mixtures to form GUVs.⁴⁵

The causes of such compositional heterogeneity are important to discuss in the context of PLB systems. During proteoliposome preparation, lipids and peptide are mixed in organic solvent that is then evaporated away to allow for hydration and MLV formation. While lipid components are highly soluble in chloroform, they are found to de-mix upon solvent evaporation, inducing macroscopic phase segregation.^{46, 47} In addition, high cholesterol content of bulk mixtures can cause cholesterol crystallization during drying.⁴⁷ Thus, the composition of MLVs formed during hydration of dry films depends on the region of the film hydrated. One might contend that sonication would robustly homogenize the mixture during SUV formation, but this has been shown not to be the case,⁴⁶ suggesting that MLVs have tremendously de-mixed compositions prior to SUV formation. Finally, one might suppose that in the last step of PLB preparation, where vesicles fuse to particle surfaces, dispersion of SUVs and coordinated fusion would better mix lipid components, but since only ~1000 SUVs of 100 nm diameter are required for fusion to a 5 μ m-sized sphere, where suspensions of sonicated vesicles contain on the order of 10^{12} – 10^{13} SUVs. From samples that contain $\sim 10^6$ silica spheres, this would result in $\sim 10^9$ vesicle fusion events, asserting that 0.01–0.1% of the total vesicle population fuse to microsphere surfaces to give PLBs. Furthermore, the kinetics of fusion are dependent on vesicle composition and size, where SUVs with more mobile components and greater elasticity will rupture and fuse rapidly after adsorption, but less mobile components require a significant amount of vesicle adsorption before rupture is initiated.^{22, 46} This was probed in a recent spherically-supported biomembrane study using fusion with different sizes of starting lipid vesicles that resulted in differences in organization of lipids and shapes of co-existing domains.³⁹ Taken together, SUVs here are considerably heterogeneous and a difference in the rate of vesicle fusion further deters homogenization, leading to PLBs with different compositions within the L_d/L_o co-existence region and fractions of L_d/L_o content.

Flow Cytometry and PLB Sorting

To gain better control of the lipid phase content of PLBs, these microspherical systems enable flow cytometry^{37, 38} in concert with fluorescence activated cell sorting (FACS). This widespread technology provides a facile method for purification that is not possible with GUVs, proteoliposomes or other intact biomembrane systems. This FACS method can be used to isolate populations of individual PLB assemblies with differing levels of L_d fraction based on scattering and fluorescence channels. Furthermore, PLB aggregates and biomembrane debris can be eliminated with FACS purification. This is especially important for MP assays as enzymes and receptors can remain highly active in this debris, skewing results. PLBs were passed through a FACSAria II Cell Sorter set to detect our FITC-labeled peptide and either DiI or QD705. In this proof-of-concept study, the FACS conditions were set at standard high speed cell sorting conditions, designed to collect ~50,000 PLBs into a ~1 ml volume from a starting PLB sample with $\sim 10^7$ PLBs/ml in less than an hour.

To quantitatively examine phase-composition based sorting, we first used labeling scheme III of Figure 1, based on the DiI lipid reporter labeling the L_d phase and the

$K_3A_4L_2A_7L_2A_3K$ -Lys(FITC) α -helical peptide distributed into both the L_o and L_d phases. The DiI labeled phase separation unsorted sample is displayed as the full 2D cytofluorogram of forward scatter channel versus the DiI channels, with the corresponding adjunct histograms shown on the X and Y axes in gray. We note that impurities and multi-PLB aggregates are widely ranging, yet the band in forward scatter from single PLB assemblies is evident as indicated by the boxed region in panel 3A. In Figure 3, panel B, the DiI channel signal was gated into Low, Mid, and High subsets indicated by the boxed region 2D cytofluorogram in Figure 3, panel B was gated by the rectangular region indicated in (A), with sorting parameters of Low-, Mid-, and High- L_d PLBs indicated by the boxed regions. Analysis of CLSM imagery was used to characterize the L_d fractions of sorted PLBs. From $N = 50$ PLBs pre-sorted PLBs had an average fraction L_d phase coverage above 0.7 (Table 1), skewing the available distribution of domain coverage to sort from. And due to the previously mentioned heterogeneous dispersion of L_d phase probes prior to sonication, the distribution of DiI intensities that were expected to correspond to a certain amount of L_d phase on each PLB becomes increasingly convoluted. The average fraction L_d measured by confocal half-projection analysis for Low, Mid, and High DiI groups is shown in Table 1. As shown in figure 3 C, we see significant differences (ANOVA $p = 4 \times 10^{-6}$) among the average fraction L_d values of the four groups examined from one-way ANOVA. With Tukey's post-hoc analysis, Low DiI is significantly different from each of the three other sample groups ($p \ll 0.05$), though no statistical differences were observed between the Pre-Sorting, Mid DiI and High DiI groups. This is easily explained by the Pre-Sorting population's high average fraction L_d ; to separate PLBs with average DiI intensity (and hence fraction L_d) from higher values would be necessarily difficult. Acknowledging this result, lowering the bulk DiI concentration could decrease the average presorting fraction L_d and improve the sorting efficiency of low, medium, and high levels of DiI intensities.

To further understand the sorting output and resulting L_d coverages in DiI sorted groups, the total fluorescence signal per PLB, i.e. fluorescence raw integrated density (RID), was measured by sumprojections from confocal Z-stacks (Figure 3, panel D). By considering the total fluorescence per PLB, as opposed to the relative area of lipid phases, we can better comprehend the functioning of flow cytometric detection and the variance in lipid reporter density per PLB, regardless of the fraction of PLB membrane that is labeled. The average RID per microsphere for Pre-Sorting, Low, Mid, and High DiI groups are shown in Table 1. Interestingly, the Pre-Sorting average RID is 1.5-fold higher than that of the High DiI group. There are two possible explanations for this: 1) fluorophores bleach during flow cytometry and FACS, or 2) a small but significant amount of lipids and tracer may desorb from the particle interface due to shear forces produced from the cytometer tube wall. Because of this, the Pre-Sorting group was not included in statistical analysis. Differences between the sorted groups are clear ($p = 5 \times 10^{-9}$), with significant post-hoc differences when comparing Low DiI to Mid DiI ($p = 3 \times 10^{-6}$) and Low DiI to High DiI ($p = 2 \times 10^{-8}$), but not when comparing Mid to High DiI ($p = 0.55$). Furthermore, a dual trend of increasing both fraction L_d and RID moving from Low to High DiI is apparent in the table. What separates the RID analysis from fraction L_d coverage is an encouraging decrease in the RID standard deviation and standard error of roughly 2-fold between Pre-Sorting and sorted groups. These results indicates that, on the basis of fluorescence per PLB detected by the cytometer, gating and

sorting has higher success, further highlighting the limitation of heterogeneous dye concentrations per unit area of L_d phase. Finally, lowering the cytometer flow and sorting rate to yield lower standard deviations in sorted PLBs comes at the expense of speed and causes excessive dilution, making comparison with CLSM data more difficult. Thus, further optimization of sorting conditions beyond the standard high speed cell sorting used is the subject of a larger investigation beyond proof-of-principle.

Flow cytometry and sorting were performed also on QD705:biotinyl-cap-PE L_d -labeled PLBs. The gating criteria for QD705- L_d -labeled PLB selection and confocal images of sorted microspheres are shown in Figure 4. Single PLBs were first isolated against multiples and/or aggregates by gating the largest discrete band at the bottom range of forward scattering (Figure 4 A). Comparing Figures 3A and 4A we note that that using streptavidin-QD705 to label the L_d Phase gave a much different level of PLB multimers and other impurities that need to be removed by FACS based purification. From the cytofluorogram in 4A, the sample was gated by the rectangular region indicated, with sorting parameters of Low-, and High-Qd PLBs indicated by the boxed regions. These PLBs were sorted into separate populations that were then imaged alongside the pre-sort group. Resulting confocal images were assessed for fractional coverage of L_d domains. Examining liposome-microsphere fusion from a bulk standpoint, one would expect the mean lipid phase coverage to be 50% L_d/L_o with a wide standard deviation, given the lipid mixture starting conditions of 1:1 DOPC to DPPC. This appears to be the case, as the average fraction L_d for the pre-sort group was 0.44 ± 0.06 with a standard deviation of 0.35 (as shown in Table 2). Also expected was the pre-sort group scatter plot distribution (Figure 4, D) displaying a full range of L_d content from the analysis $N = 34$ PLBs.

The low-QD705 gated group shown in Figure 4 ideally represents PLBs displaying L_d fractions below 0.5. After sorting this population, analysis of $N = 28$ PLBs revealed an average fraction L_d of 0.15 ± 0.04 . The scatter plot of this group with the accompanying histogram (Figure 4, C) shows that the sorted PLBs have L_d fractions largely below 0.4, demonstrating that sorting PLBs can enrich sub-populations of estimated L_d/L_o content. The average RID per microsphere for Pre-Sorting, Low Qd, and High Qd groups are shown in Table 2. The Pre-Sort Qd average RID is slightly lower than that of the High Qd group. Differences between the sorted groups are clear ($p=2 \times 10^{-8}$), with significant post-hoc differences when comparing Low Qd to High Qd ($p = 4 \times 10^{-6}$). Furthermore, a dual trend of increasing both fraction L_d and RID moving from High to Low Qd is apparent in the table. While it can be reasoned that the sorted particles represent roughly 40% of the original PLB population in terms of L_d content, the range of QD705 reporter that was gated in the FACS machine is clearly much smaller than 40% of the full distribution. A variable likely at play here is the heterogeneous QD705 labeling densities per unit area L_d phase previously mentioned. Nevertheless, this sorting methodology was successful in positioning a MP analogue in a high L_o content micro-environment in the PLB format, potentially suitable for designing assays based on millions of intact biomembrane assemblies of this type. One can envision using this method to load lipid-composition sensitive MPs such as p-glycoprotein,^{48, 49} γ -secretase,^{50,51} or growth factor receptors⁵² into cholesterol-rich microdomains to probe their function in bilayers of larger hydrophobic thickness relative to liquid disordered L_d PLB controls (via domain based FACS). We note that it is likely to be

very challenging to reconstitute MPs into proteoliposomes and GUVs with predominantly L_o content.

Imaging and $K_{p,app}$ Determination

The partitioning of membrane peptides and proteins is a significant topic in the study of MP functioning, as several classes of MPs are affected by or sequester to ordered lipid domains.^{8, 12} Measurement of α -helical peptide-FITC partitioning between ordered and disordered phases demonstrates the capability of these microsphere-biomembrane systems for the study of phase-dependent MP localization and activity. Spectral imaging was used to measure peptide interphase partitioning, as employing a single PMT detector for imaging across the FITC and QD705 emission spectra ensures the most consistent fluorescence readings. A lambda-coded 3D reconstruction (Figure 5, panel A) displays the coexistence of ordered and disordered phases with QD705 patches and peptide localized to both phases. Shown in Figure 5, panel B is a representative equatorial slice at peak QD705 emission. The average peak FITC intensity from two ROIs in each phase (phase indicated by the relative intensities of QD705 signal) were used to calculate the apparent peptide partitioning coefficient, $K_{p,app}$. $K_{p,app}$ is defined here as

$$K_{p, app} = \frac{\langle I_{L_d} \rangle}{\langle I_{L_o} \rangle}$$

where $\langle I_i \rangle$ is the average peak FITC fluorescence intensity measured in lipid domain i . This formalism is consistent with prior work in the field.³⁵ Measurements of $K_{p,app}$ are centered on an average value of 0.89 ± 0.06 . $K_{p,app}$ values were found to be uncorrelated to the level of total peptide loading per PLB over the range studied, as indicated by the total FITC fluorescence (raw integrated density in AU) per PLB (outlined in supplemental data Figure S1) From this it can be assumed that FITC quantum yield is consistent across L_d and L_o phases. For reference, the DiI L_d -specific probe partition coefficient was determined to be $K_{p,app,DiI} = 3.12 \pm 0.33$, as expected very highly favoring the L_d phase. Moreover, $K_{p,app}$ measurements were consistent when analyzed in a separate analogous system where quantum yield variables were removed by external peptide labeling with biotin-Streptavidin-QD705 (Figure 1: Labeling Scheme I), displayed in Figure 6. In this case, DiI was used to label L_d phase, and the α -helical peptide FITC partition coefficient was measured to be $K_{p,app}(K_3A_4L_2A_7L_2A_3K\text{-Lys(FITC)}) = 0.91 \pm 0.06$ in this experiment. The biotinylated- α -helical peptide is concentrated slightly in the L_o cholesterol-rich phase, indicating that control of phase separation in PLB systems could provide a new biomimetic route to manipulate mobile ligand surface density.

The mean $K_{p,app}$ indicates a slight favoring for $K_3A_4L_2A_7L_2A_3K\text{-Lys(FITC)}$ localization within the DPPC and cholesterol-rich L_o phase. Bearing in mind the mechanisms of liposome fusion to microspheres and the mobility of vesicle components during fusion and thereafter (as the system approaches equilibrium), it was of interest to measure peptide diffusivity within each lipid phase. FRAP was performed on equatorial planes of PLBs within the respective phase boundaries. Compared to the pure DPPC melt-transition temperature of 41 °C (Avanti Polar Lipids Technical data), samples were incubated at 37°C,

ensuring that peptide mobility within the L_o domain could be detected by FRAP. By imaging QD705 signal in a pre-FRAP image prior to each FRAP sequence, regions of L_d and L_o could be distinguished during diffusivity analysis. From $N = 5$ PLB constructs taken from selected equatorial z-sections, D_{eff} was measured to be $0.036 \pm 0.005 \mu m^2/s$ in L_d regions and $0.014 \pm 0.003 \mu m^2/s$ in L_o regions ($p = 0.008$). The average mobile fraction was similar between the two phases: 0.78 ± 0.09 L_d regions and $0.79 \pm 0.06 \mu m^2/s$ in L_o regions, ($p = 0.9$). These values are in agreement with our earlier measurements of α -helical peptide-FITC diffusivity within DOPC supported biomembranes.⁵³ Furthermore, this L_d D_{eff} value is within the same order of magnitude with the finding of Merzlyakov et al. for a 33-residue length single-helix peptide that was comprehensively studied in a range of conditions.⁵⁴ Other higher values for single helix peptide diffusivities have been reported in bilayer systems using different lipid species and with different levels of peptide loading, as well as helix length or overall peptide length.^{55, 56} The ~ 2.6 fold reduction in diffusivity of the peptide in the L_o phase in this work is consistent with findings from pulsed field gradient-NMR diffusion experiments that lipid mobility decreases 2- to 10-fold in the ordered phase.⁵⁷⁻⁶¹ To our knowledge there have been no other reports of diffusivities of similar single α -helix peptides in the L_o phase thus far.

Several references point out the partitioning of helical peptides and MPs toward detergent soluble or L_d domains within phase-separated membranes in GUVs^{8, 12} though the issue may be confounded by changes in peptide or protein conformation (e.g. tilting, stretching and oligomerization) to alleviate hydrophobic length mismatch; one may even consider the opposite scenario of the protein's influence on remodeling the local lipid environment.⁶² It is important to point out that the path to phase-separated intact bilayers is quite different when comparing these PLB systems to GUVs constructed by electroformation. Even though both start with MP-loaded lipid films, the PLBs are constructed from the fusion of liposomes formed from ultrasonication that is concomitant with high levels of hydrated lipid bilayer mixing. Effectively, any potential interphase diffusion barriers have likely been overcome during the fusion leading to PLB formation.

Here, a 23-residue helical peptide appears to partition into both phases in nearly equal amounts, with a slight favoring of the L_o phase. At a bulk concentration of 4 mol%, peptide oligomerization is certainly possible, but indistinguishable from its partitioning within a given phase based on fluorescence imaging alone. What differentiates the membrane peptide featured in this work is the addition of FITC at the N-terminal lysine (ϵ -amine group). Once conjugated, fluorescein is sure to impart increased hydrophobicity to the N-terminal portion of the molecule, in addition, our peptide is highly asymmetric with respect to the helical axis. This may effectively increase the peptide's hydrophobic length, allowing it to better partition into the L_o phase, as the ordered domain is known to have longer hydrophobic lengths.⁸ Experiments probing the hydrophobic thicknesses of DOPC vs. DPPC membranes report values of 26.8 vs. 27.3 Å⁶³ while the hydrophobic core of the peptide sequence used here is 27.0 Å based on the number of α -helix residues. Also, cholesterol is known to increase the hydrophobic thickness of membranes,^{26, 64} promoting increases in both phases, but likely more so in the L_o phase where cholesterol is more abundant. The width of FITC can be estimated to be ~ 0.8 nm based on C-C bond lengths, and so its addition may increase the hydrophobic component of the peptide, better matching the thicker of the two lipid

phases. The amount of L_o partitioning has been controlled by changing MP hydrophobic domain length in two recent examples in the literature.^{65, 66}

CONCLUSIONS

We have demonstrated that a highly heterogeneous population of phase-separated biomembranes with integrated membrane protein analogues can be compositionally sorted by flow cytometry when fused to microsphere support substrates. Sorting was not highly efficient as shown by overlapping measurements of phase content across sorted groups, leaving sizeable room for improvement. Nevertheless, this technique allows for immediate application to biochemical assays involving domain-influenced protein functioning in biomimetic membrane environments. Moreover, interphase peptide partitioning was measured for the first time by spectral imaging, giving highly resolved fluorescence characterization and improving on quantitative microscopy. FITC-labeled peptides were found to associate in both phases with slight preference for the ordered lipid phase, due to its increased hydrophobic length by fluorophore coupling. Future attention can be directed at optimizing these systems for increasing resolution of population sorting and incorporation of different MP or peptide species.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

FACS	fluorescence-activated cell sorting
CLSM	confocal laser-scanning microscopy
FRAP	fluorescence recovery after photobleaching

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Figure 1. PLB labeling schemes

Panel A denotes Labeling Scheme I: the L_o phase contains Biotinyl- $K_3A_4L_2A_7L_2A_3K$ -Lys(FITC) bound to Streptavidin Qd 705 and the L_d phase contains Biotinyl- $K_3A_4L_2A_7L_2A_3K$ -Lys(FITC) bound to Streptavidin Qd 705 and DiI lipid tracer. Panel B denotes labeling scheme II: the L_o phase contains $K_3A_4L_2A_7L_2A_3K$ -Lys(FITC) and the L_d phase contains DOPE-cap-biotin bound to streptavidin Qd 705. Panel C denotes labeling Scheme III: the L_o phase contains $K_3A_4L_2A_7L_2A_3K$ -Lys(FITC) and the L_d phase contains $K_3A_4L_2A_7L_2A_3K$ -Lys(FITC) C-12 DiI lipid tracer.

Figure 2.

3D reconstruction of $K_3A_4L_2A_7L_2A_3K_2$ -FITC α -helical peptide display and phase partitioning in 1:1 DOPC:DPPC (L_d : L_o) with 30% cholesterol and 4% peptide-FITC. Panel A is a zoomed out 2-channel 3D CLSM reconstruction of >50 PLBs, displaying the phase heterogeneity and PLB aggregation/defects. As indicated in labeling scheme I of figure 1, L_d phase is labeled by C12-DiI lipid reporter (red). The biotinyl- $K_3A_4L_2A_7L_2A_3K_2$ -FITC α -helical peptide in the L_d and L_o phases is labeled by bound QD705-streptavidin (green (false color)). Panel B, top graph, is a histogram of the raw DiI (L_d) channel integrated density per PLB obtained from the CLSM indicating the heterogeneity of L_d phase. For comparison, the bottom graph of panel B is a histogram of the StrepAv-Qd705:Biotinyl- $K_3A_4L_2A_7L_2A_3K$ -Lys(FITC) (L_d / L_o) channel integrated density per PLB indicating the relative homogeneity of the α -helical peptide loading into the PLBs. Panel C is a zoomed in 2-channel 3D CLSM reconstruction, also displaying of the phase heterogeneity and PLB aggregation/defects. As indicated in labeling scheme II of figure 1, L_d phase is labeled by DOPE-cap-biotin bound to QD705-streptavidin (red). The $K_3A_4L_2A_7L_2A_3K_2$ -FITC α -helical peptide in the L_d and L_o

phases is labeled by intrinsic FITC (green). The microsphere diameters are $\sim 4.9 \mu\text{m}$, providing a reference of image scale.

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Figure 3.

Sorting of Low-, Mid-, and High- L_d content PLBs L_d -labeled by DiI (Labeling Scheme I). (A) The full 2D cytofluorogram of the histogram of forward scatter channel versus the DiI channel. Single points come from individual PLBs. The single PLB assembly region band in the FSC is delineated by the box shown by dotted lines. The adjunct histograms are projected in grey on the Y and X axes. (B) The Flo-Jo 10.1 2D cytofluorogram gated by the rectangular region indicated in (A), with sorting parameters of Low-, Mid-, and High- L_d PLBs indicated by the boxed regions. (C) CLSM-detected distribution of phase coverage in these Pre-Sorted and sorted groups, as measured by half-projection calculations. (D) CLSM-detected distribution of the average raw integrated density in the Pre-Sorted and sorted groups, as measured by half-projection calculations, showing scatter points with accompanying histograms.

Figure 4.

Sorting of low- L_d content PLBs labeled by streptavidin-QD705 bound to biotin-cap-PE in L_d phase (Labeling scheme II). (A) The full 2D cytofluorogram of the histogram of forward scatter channel versus the QD705 channel. Single points come from individual PLBs. The single PLB assembly region band in the FSC is delineated by the box shown by dotted lines. The adjunct histograms are projected in grey on the Y and X axes. (B) The Flo-Jo 10.1 2D cytofluorogram gated by the box in A, with sorting parameters of Low- and High-Qd PLBs indicated by the boxed regions. Panel B shows the cytometry sorting criterion used, with sorting based on PLB size (Forward scatter channel), and QD 705 fluorescence per PLB assembly, indicated by the boxes overlaying the 2D cytofluorogram. Adjunct histograms are displayed in gray on the top Y and right X axis. (C) CLSM-detected L_d phase coverage fraction in the Pre-Sorted and sorted groups, as measured by half-projection calculations, showing scatter points with accompanying histograms. (D) CLSM-detected average raw integrated density in these Pre-Sorted and sorted groups, as measured by half-projection calculations.

Figure 5.

Peptide phase partitioning observed by spectral imaging (Labeling Scheme II). (A) Lambda-coded 3D confocal reconstruction of phase-separated PLBs. Emission at 525 nm (green) shows peptide-FITC display in regions both co-localized with and isolated from 705 nm emission (red) signal from QD705 (L_d). (B) Spectral imaging readout for an example equatorial slice of peak QD705 emission displaying non-uniform L_d phase coverage (Labeling Scheme II) used to measure FITC-QD705 emission spectra within individual phases. $K_{p,app}$ was measured to be 0.89 ± 0.06 , calculated by ratioing average peak FITC emission from ROIs in L_d and L_o regions (white boxes). (*) FITC peak, (**) QD705 peak.

Figure 6.

Phase concentration of peptide ligand display in phase separated proteolipobeads. Spectral profile of DiI- L_d -labeled sample with QD705 secondary labeling of peptide-FITC (Labeling Scheme I). Peptide-FITC partitioning coefficient was measured to be 0.91 ± 0.06 in this experiment, consistent with the previous measurement. $K_{p,app}$ was calculated by ratioing average peak FITC emission from ROIs in L_d and L_o regions (white boxes). (*) FITC peak, (**) QD705 peak.

Table 1

Table 1 is a comparison of fraction disordered phase lipid and raw integrated density between Pre-sorting and sorted PLB groups labeled by DiI. Standard deviations and errors are included to highlight the relative distribution tightening of fraction L_d and RID pre- and post-sorting.

	Average Fraction L_d	Standard Deviation	Standard Error	Average Raw Integrated Density ($\times 10^{-7}$)	Standard Deviation ($\times 10^{-7}$)	Standard Error ($\times 10^{-7}$)
Pre-Sort	0.74	0.25	0.03	7.1	3.2	0.45
Low DiI	0.54	0.25	0.04	2.9	1.6	0.22
Mid DiI	0.72	0.19	0.03	4.5	1.7	0.24
High DiI	0.76	0.20	0.03	4.9	1.6	0.23

Table 2

Table 2 is a comparison of fraction disordered phase lipid and raw integrated density between Pre-sorting and sorted PLB groups labeled by streptavidin-Qd705:PE-cap-Biotin Ld phase labeling. Standard deviations and errors are included to highlight the relative distribution tightening of fraction L_d and RID pre-and post-sorting.

	Average Fraction L _d	Standard Deviation	Standard Error	Average Raw Integrated Density (×10 ⁻³)	Standard Deviation (×10 ⁻³)	Standard Error (×10 ⁻³)
Pre-Sort	0.44	0.44	0.06	101.7	118.4	20.0
High Qd	0.55	0.55	0.05	109.1	108.9	20.9
Low Qd	0.15	0.15	0.04	16.7	13.7	2.5