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Observation of Inhomogeneity in the Lipid Composition of Individual Nanoscale Liposomes

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Supporting Information

ABSTRACT: Liposomes, or vesicles, have been studied extensively both as models of biological membranes and as drug delivery vehicles. Typically it is assumed that all liposomes within the same preparation are identical. Here by employing pairs of fluorescently labeled lipids we demonstrated an up to 10-fold variation in the relative lipid composition of individual liposomes with diameters between 50 nm and 15 μm. Since the physicochemical properties of liposomes are directly linked to their composition, a direct consequence of compositional inhomogeneities is a polydispersity in the properties of the individual liposomes in an ensemble.

Liposomes have been studied extensively over the past decades both as models of biological membranes[1] and as biocompatible nanocontainers for drug delivery applications.[2] The majority of these experiments have implicitly assumed that all liposomes in an ensemble are identical. However recent measurements performed at the level of single liposomes[3] revealed the existence of intrinsic intrasample heterogeneities[3a,c,4] that were otherwise averaged out in ensemble experiments. Here by employing fluorescently labeled lipids we demonstrated significant inhomogeneity in the relative lipid composition of individual liposomes with diameters between 50 nm and 15 μm. Since the physicochemical properties of liposomes are directly linked to their composition a direct consequence of compositional inhomogeneities is a polydispersity in the properties of the individual liposomes in an ensemble.

To investigate a possible inhomogeneity in the lipid composition of individual liposomes we labeled liposomes with various pairs of fluorescent lipids and lipid analogues. We then isolated individual liposomes by tethering them through streptavidin−biotin coupling on a passivated glass surface at low densities (Figure 1A).[3d] Imaging with confocal fluorescent microscopy allowed us to monitor single liposomes in a high-throughput manner, $\sim 10^3$ per frame. Each liposome was imaged sequentially in two fluorescent channels. The ratio of the integrated intensities of the two channels gave us a measure of the molar ratio of the two labels that was independent of the size of the liposomes.

Assuming a homogeneous lipid composition for every liposome the ratio between the two labels would be constant. However, as can be visually inspected in the surface plots of the fluorescent intensity of DHPE-Atto^{633} and C_{16}-fluorescein in Figure 1B and C, we measured great variations in the intensity ratios between liposomes. The molar ratios of the two labels in liposome 1 and 2 were 0.21 and 1.40 respectively and thus differed by a factor of $\sim$7.

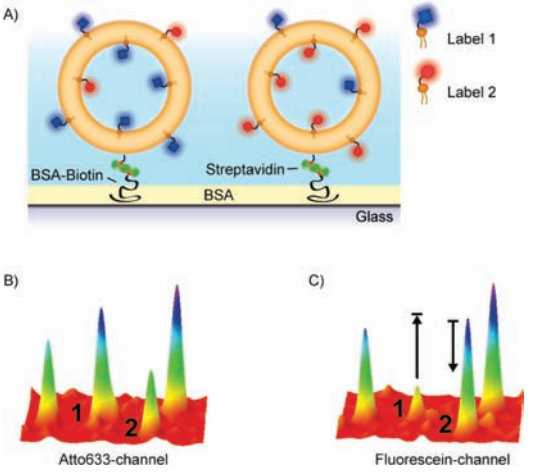

Figure 1. Assay for measuring inhomogeneity in the lipid composition of single liposomes. (A) Liposomes composed of DOPC:DOPG (9:1) and $\sim$1% of two fluorescently labeled lipid analogues were tethered to a passivated glass surface through a biotin-streptavidin coupling and imaged sequentially with fluorescence microscopy. (B, C) Surface plots of fluorescence intensity from the Atto^{633} and fluorescein channel respectively for a typical region of interest (3.2 × 4.9) μm^2. Liposomes 1 and 2 (see arrows) have a significantly different ratio of labeled lipid components.

To quantify this inhomogeneity in a rigorous manner we plotted a histogram of the integrated intensity ratios for a few thousand single liposomes (Figure 2A, red). We then fitted this histogram with a Gaussian function and calculated the *Degree of Inhomogeneity* (*DI*) as the standard deviation divided by the mean of the fit. To quantify the error introduced in *DI* from image acquisition and data treatment we imaged the same liposomes multiple times, defocusing and refocusing before each acquisition, and determined a *DI* of 0.09 ± 0.01 (Figure 2A, blue). Errors due to statistical uncertainties in the redistribution of labels in the smallest liposomes were calculated with Poissonian statistics to be of approximately equal value (see Supporting Information (SI) Figure S1). The errors in *DI* are typically calculated as the standard deviation between three separate liposome preparations. Thus due to measurement errors 32% of the liposomes will appear to have more than a 9% difference in their molar ratio.

Next we repeated these measurements for the two different labels and measured a *DI* of 0.33 ± 0.04 which is almost four

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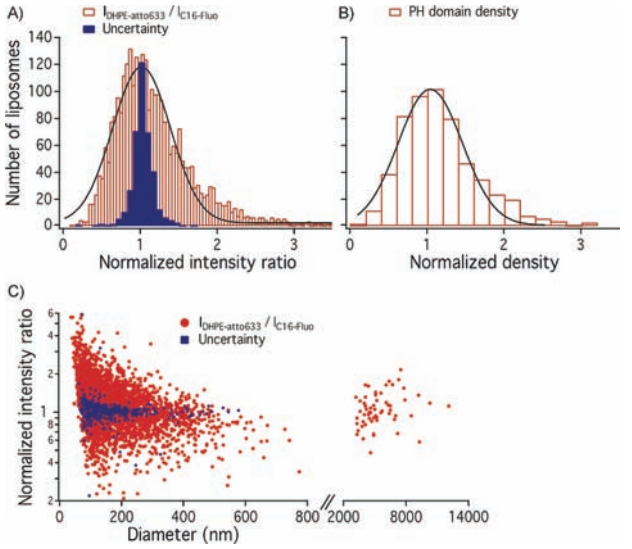

Figure 2. Quantification of inhomogeneity in the lipid composition of single liposomes and its consequences on protein binding. (A) Normalized intensity ratios of DHPE-Atto^{633} and C_{16}-fluorescein for single liposomes plotted as a histogram (red) and fitted with a Gaussian function (black). Uncertainty arising from errors in image acquisition and data treatment is depicted in blue. (B) Histogram of PH domain density on single liposomes containing PiP_2. (C) Normalized intensity ratio as a function of liposome size.

times greater than the experimental uncertainty and hence indicates the presence of significant inhomogeneities in the composition of different single liposomes (Figure 2A, red). Thus for this system 32% of the liposomes in the population will differ by more than ~33% from the mean molar ratio of the ensemble.

To provide an independent verification of the aforementioned results we employed the PiP_2 lipid-binding Pleckstrin Homology domain of Centaurin β2 that was labeled with Alexa^{488} (PH domain).[4a] The PH domain was incubated at saturation conditions (1.75 μM) on single liposomes that were labeled with DHPE-Atto633 and contained 5 mol % PiP_2. The density of the PH domain on individual liposomes was thus used as an indirect measure of the density of PiP_2. Indeed Figure 2B shows a *DI* of 0.40 and normalized densities varying by an order of magnitude (~0.2 to ~2.5) between individual liposomes in the same sample. This experiment demonstrates how the inhomogeneity in lipid composition can propagate and influence the protein binding efficiency of membranes, thus having direct implications on the protein densities measured in nanoscale studies *in vitro*.

Liposome diameter influences both the signal-to-noise in our images and the number of labels per liposome and thus statistical fluctuations in the number of labels. To uncouple this influence we plotted densities as a function of diameter in Figure 2C, where liposome sizes were calculated as described previously by calibrating the total intensity per liposome that scales with the total membrane area.[5] Figure 2C demonstrates a reduction of *DI* for larger liposomes, which however maintain variations in their composition that are three times greater than the experimental uncertainties (giant unilamellar vesicles (GUVs) with diameters 3−12 μm had a *DI* of 0.25; see Figure S5). Indeed, GUVs within the same preparation are known to exhibit different phase-transition behavior[1c] and our work suggests that this is due to variations in their lipid composition.

As described previously[3d,4a,5] one can use the number of fluorescent labels to deduce the size of individual liposomes (Figure 2C). Since the average lipid variation is constant with liposome size, compositional inhomogeneity will not introduce any systematic bias in the determination of size but will introduce an uncertainty ≤15% for ~70% of the liposome population (see SI).

In Figure 2, Atto^{633} and fluorescein were conjugated on DHPE and C_{16} respectively. To examine if the nature of the lipid anchor had an influence on the interliposome inhomogeneities we placed these two labels on the same lipid moiety (DHPE). Figure S3A shows that the *DI* in this case was reduced to 0.25 ± 0.02, approximately three times greater than the measurement uncertainties, indicating that the lipid species can influence the inhomogeneities in lipid composition. Choosing an additional pair of amphiphilic fluorescent probes with great structural similarity (DiD and DiO) did not change significantly the results (*DI* 0.21 ± 0.02; see Figure S3B). These observations has been reproduced for different lipid ratios (see Figure S2) further validating that compositional inhomogeneity is not an artifact of self-quenching nor does it originate from Poissonian statistical uncertainties. Preparation of liposomes through detergent dilution, a slow equilibrium-like process, showed very similar trends for *DI* (Figure S6) suggesting that the observed inhomogeneities are not due to kinetically trapped states induced by rehydration of the dried lipid films. The observations made for a variety of different lipid labels, lipid compositions, and preparation methods suggest compositional inhomogeneity to be a general phenomenon present in liposome systems.

In this communication we show how fluorescent labeling of pairs of lipid analogues can provide information about the relative lipid composition of single liposomes with diameters down to ~50 nm. This new method revealed variations of up to an order of magnitude in the relative lipid composition of individual liposomes within the same preparation. These results are particularly relevant for the growing number of studies that manipulate and observe single liposomes[3] that if made from more than one lipid component will exhibit a significant variation in their lipid composition and their physicochemical properties (see e.g. Figure 2B) thus introducing an additional source of "noise" to single liposome measurements. For the latter reasons it would be exciting to investigate if compositional variations pertain to nanoscale assemblies in vivo, e.g., synaptic or endocytic liposomes[6] or protein assemblies.[7] These results are also relevant for many large-scale applications of liposomes in drug delivery and biotechnology since a polydispersity in the properties of individual liposomes in an ensemble will translate to "broadening" of the response of the ensemble to any given perturbation, e.g., drug release as a function of temperature change.[2a] Identifying and characterizing intrasample compositional variations will hopefully open up new routes for control and optimization.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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