Phospholipids Diffusion on the Surface of Model Lipid Droplets

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Abstract

Lipid droplets (LD) are suborganelles localized in the membrane of the Endoplasmic Reticulum (ER) that play an important role in metabolic functions. They consist of a core of neutral lipids surrounded by a monolayer of phospholipids and proteins resembling to an oil-in-water emulsion droplet. Many studies have been focused on the biophysical properties of these LDs. However, despite numerous efforts, we are lacking information on the mobility of phospholipids on the surface of LDs surface, although they may play a key role in the protein distribution. In this article, we developed a microfluidic setup that allows the formation of a triolein-buffer interface decorated with a phospholipid monolayer. Using this setup, we measured the motility of phospholipid molecules by performing Fluorescent Recovery After Photobleaching (FRAP) experiments for different lipidic compositions. The results of the FRAP measurements reveal that the motility of phospholipids is controlled by the monolayer packing decorating the interface.

Keywords: `Phospholipid, Lipid droplet, Diffusion, FRAP`

1. Introduction

Lipid droplets are suborganelles present in the Endoplasmic Reticulum (ER) membrane [1, 2]. They attract many interest due to their role in several diseases

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like cancer and metabolic problems [3, 4, 5]. One of their functions is to store lipids and to regulate their consumption based on mechanisms that are not yet understood [6]. Lipid droplets (LDs) are composed of a core of neutral lipids and are surrounded by a phospholipid monolayer [7, 8]. Their biogenesis is assumed to occur in three consecutive steps [2, 9]. First, neutral lipids are synthesized in the ER membrane. Then, they accumulate and segregate into the ER bilayer core to form a droplet with a lens shape [2, 10, 11]. And finally, they grow through the accumulation of lipids until budding and sometimes pinch-off from the ER membrane [2, 12, 13, 14, 15]. Many of the biophysical properties of LDs have been investigated over the last decade using model systems [6]. As example, droplet interface bilayers (DiB), advanced free-standing bilayer (AFB), and giant unilamellar vesicles (GUVs) have been extensively used to reconstitute artificial and natural LDs in such lipid membranes [16, 17, 18, 19, 20, 21]. After successful reconstitution of LDs in a bilayer, the morphology of LDs was studied as function of the phospholipidic composition of the DiBs, GUVs or AFB systems [18, 19, 20]. It has been shown that the shape of LDs in a bilayer is determined by the balance of surface forces at the contact line between the embedded-LDs and the bilayer [18, 19, 20]. Thus, the associated contact angle of LDs can be predicted using wetting theory [11, 22]. Accordingly, numerical simulations based on continuum models were able to confirm many experimental results obtained on microscopic LDs [15, 18, 23, 24, 25, 26, 27].

In addition to the equilibrium shape determined by a force balance, non-equilibrium phenomena related to LDs have also attracted attention in recent years. A key example is protein partitioning, which is used to understand the dynamic distribution of specific proteins between the monolayer covering the LD and the bilayer in which the LDs are embedded [6, 16]. Indeed, protein partitioning seems highly dependent of the physical properties of the lipid monolayer (packing, fluidity) that surrounds the LD surface [18, 17]. However, to the best of our knowledge, there are no experimental measurements on phospholipid properties such as motility at the LD surface. One reason for this lack of knowledge could be the small size of the reconstituted LDs in the artificial systems

(DiB, AFB, GUVs), which make FRAP experiments and extraction of precise diffusion coefficients challenging [17, 28]. In consequence, there are few experimental studies in the literature that address diffusion [24]. Interestingly, this problem is not limited to experimental studies but, also, occurs in theoretical studies with numerical simulations [18, 25, 29].

In this article, we experimentally investigate the motility of phospholipids covering the interface between an aqueous and an oily phase. For this purpose, we have developed a microfluidic setup that enables the formation of a triolein-buffer interface decorated with phospholipids and have performed FRAP experiments directly at this interface (Fig. 1). Here, the planar phospholipid monolayer at the triolein-buffer interface mimicks the surface of a LD. We used triolein as neutral lipid because it is an important component of the biological LD [5] and varied the molecular composition of the monolayer to assess the influence of the most common molecules that make up the LD surface in living cells. These molecules are mainly phospholipids and cholesterol. Because native biological LDs may contain cholesterol ester [30, 31], we also investigated the influence of LD-core composition on the phospholipid motility by adding some cholesterol ester (chol-ester) to the neutral lipid (triolein). We also tested the possible influence of a relevant human protein, Perilin-2 [30, 31] (PL2), on phospholipid motility with and without chol-ester in the LD core. Our measurements show that the motility of amphililic lipids is directly influenced by the packing of these molecules coating the interface.

2. Results and Discussion

Liquid droplets (LDs) originate in the ER membrane, and the phospholipid monolayer that decorates LDs is therefore composed mainly of phospholipids, sterols and proteins [33, 34, 35, 36, 37], with phospholipids making up the vast majority and proteins and sterols being present in only a small percentage. Phospholipids can be distinguished in two families, saturated and unsaturated. Accordingly, we start our discussion with the most common lipids and pre-

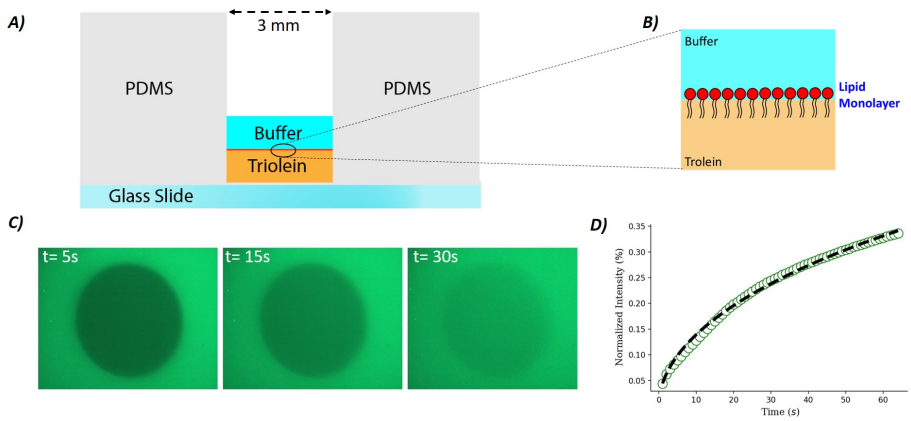

Figure 1: A) Sketch of the microfluidic device. B) Schematic of the phospholipid decorated triolein-buffer interface. C) Example of a FRAP measurement; the time $t = 0$ corresponds to the moment when the bleaching was stopped. D) Experimental intensity data (green dots) as obtained from a FRAP measurement plotted together with the Soumpasis equation fitted to these data (dashed line) [32].

sented the results sorted by lipid families: unsaturated phospholipids, saturated phospholipids, and sterols.

Unsaturated Phospholipids: DOPC, DOPE, N-DOPE, POPC, LPC. A relevant biophysical composition for a phospholipid monolayer covering LDs localized in the ER membrane can be approximated by a DOPC/DOPE mixture with a molar ratio between 1:1 and 3:1 [7, 8, 17, 18]. For completeness, we vary the composition from pure DOPC to a pure DOPE monolayer. The extracted diffusion constants are presented in Fig. 2 and Table 1. The diffusion constant of a pure DOPC monolayer is $D \approx 8\ \mu m^2 \cdot s^{-1}$, i.e. close to, or slightly slower than, the DOPC fluidity observed in giant vesicles [28, 37]. For increasing DOPE content, the fluidity monotonically increases reaching $D \approx 17\ \mu m^2 \cdot s^{-1}$ for a pure DOPE monolayer. This trend can be easily understood considering the lipid packing that results from the geometry of these two molecules. DOPC can be approximated as a cylinder, while the shape of DOPE is closer to a truncated cone [38]. Thus, the corresponding critical packing parameter $\rho = V/a_0 \cdot l$ is $\rho_{\mathrm{DOPC}} \approx 1$

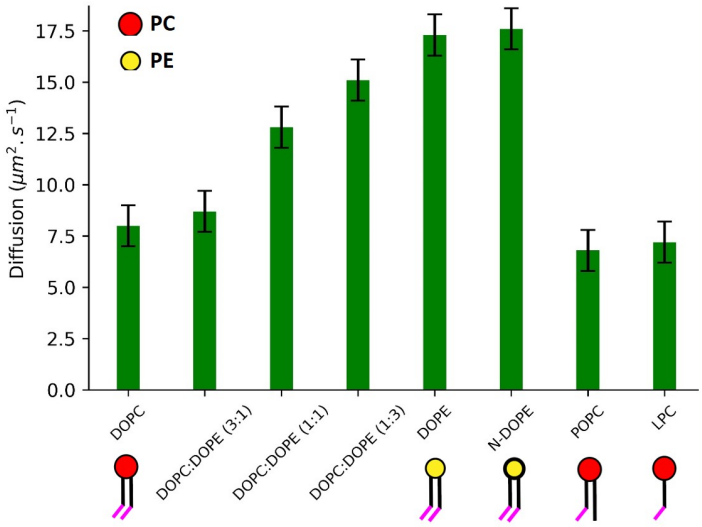

Figure 2: Diffusion constants D for several saturated phospholipids (DOPC, DOPE, N-DOPE, POPC, LPC) and mixtures of DOPC and DOPE at a triolein-buffer interface extracted from FRAP experiments. Schematic representations of the various lipids are provide for PE (red head), PC (yellow head) where pink tails indicate one unsaturation.

for a monolayer consisting only of DOPC molecules and $0.5 < \rho_{\mathrm{DOPE}} < 1$ for a monolayer consisting only of DOPE molecules [38]. For a flat interface, a DOPC monolayer obviously has a higher lipid packing than a DOPE monolayer. In consequence, at equivalent surface coverage, the average motility of a single DOPE molecule is larger than that of a single DOPC molecule. Accordingly, packing density and mobility of DOPC and DOPE mixtures interpolate between the limits of a pure DOPC and DOPE monolayer, respectively. As the increased presence of lipids at the triolein-buffer interface lowers the surface tension, differences in surface coverage can also be confirmed by differences in surface tension measured by the hanging drop method as $\gamma_{\mathrm{DOPC}} \approx 1.5$ mN/m and $\gamma_{\mathrm{DOPE}} \approx 2.3$ mN/m, for DOPC and DOPE, respectively. These surface tension values are consistent with expectations based on geometry dependent lipid packing and the experimentally observed lipid motility.

To confirm the influence of the lipid packing on lipid motility, we repeated

the experiments described above with monolayers of N-DOPE, POPC and LPC molecules. Compared to DOPE, has N-DOPE an additional methyl group in the PE head, thus this molecule is slightly less conical than DOPE but also less cylindrical than DOPC (Fig. 2). The measured diffusion constant is $D \approx 17\ \mu m^2 \cdot s^{-1}$ and surface tension is ≈ 2 mN/m for a pure N-DOPE monolayer. As expected from the N-DOPE geometry, are both diffusion constant and surface tension rather comparable to pure DOPE monolayer.

POPC has the same hydroplilic head as DOPC and the same length of the hydrophobic tail, but only one unsaturation in its tail [34, 35]. Thus, due to this little difference, a POPC monolayer is expected to present a similar packing than a DOPC monolayer [27, 33, 39, 40]. And consistently with the geometry of the molecules and the expected monolayer packing, we measured the diffusion constant and the surface tension of POPC as $D \approx 7\mu\mathrm{m}^2 \pm 1 \cdot \mathrm{s}^{-1}$ and $\gamma \approx 1.3 \pm 0.3$ mN/m, that are very similar to those of a pure DOPC monolayer, see Fig. 2.

LPC is a single tail lipid with one unsaturation in its tail and a conical geometry. The measured diffusion constant for a pure LPC monolayer of $D \approx 7\mu\mathrm{m}^2 \cdot \mathrm{s}^{-1}$ and its surface tension is $\gamma \approx 1.3$ mN/m, see Fig. 2. Thus, this LPC appears to have a motility close to POPC and DOPC. This result is a little surprising, as *a priori*, we may have expected to measure a stronger difference between a monolayer composed of lipids with a single tail or a double tail. However, the correlation between low surface tension resembling high lipid packing and low lipid motility still holds true.

Saturated phospholipids (DPPC, DSPA) and Sphingolipids (SM). As described in the previous section, the surface of LDs consists mainly of lipids that may be unsaturated or saturated phospholipids [41]. In this section, we explore the motility of saturated phospholipids (DPPC, SM) on the surface of model lipid droplets. Due to the absence of unsaturations, the packing of unsaturated (neutral) phospholipids may be expected to be denser than that of unsaturated phospholipids and so the corresponding motility is expected to be lower than

that of DOPC. However, the degree of saturation is not the only interaction that controls the monolayer packing. As example, electrostatic interactions can also strongly influence the packing of lipid monolayers. For this reason, we also explore below the mixture between saturated DSPA molecules having an negatively charged hydrophilic head group and uncharged and unsaturated DOPC molecules. In addition to saturated lipids, we will also consider the case of sphingolipids.

DPPC is a saturated lipid with a cylindrical shape that self-assembles in an highly packed monolayer [42]. The measured diffusion constant for a pure DPPC monolayer is $D \approx 5.5\ \mu\mathrm{m}^2{\cdot}\mathrm{s}^{-1}$, which is a little slower to that of unsaturated DOPC and POPC phospholipids, that are also expected to be densely packed (see, Fig. 3). The high DPPC packing at the triolein-buffer interface is also confirmed by the low surface tension, which is $\gamma \approx 1.1$ mN/m, also very similar to surface tensions values for DOPC and POPC. In addition, DPPC can also condense at room temperature, which is another reason for the reduced fluidity [43].

DSPA is a saturated double-tail lipid with a head that is negatively charged and with a conical shape. Due to electrostatic repulsion, the lipid packing of a pure PA monolayer is smaller than that of other lipids [44]. In addition to a large motility of $D \approx 20\ \mu m^2 \times s^{-1}$ of a pure DSPA monolayer, the comparatively low packing density also leads to a comparatively large surface tension at the triolein-buffer interface, which was measured to be $D \approx 2.6$ mN/m, see table 1. Mixtures of DOPC and DSPA molecules show motilities intermediate between those of a pure DOPC and a pure DSPA monolayer. Presumably due to the repulsion of the charged DSPA molecules, the measured diffusion constants are already quite large at a DSPA content of 25% and increase even further with increasing DSPA content, see Fig. 3.

Sphingolipids are a family of lipids that are synthesized in the (ER) membrane, where they can be found in low amounts, as well as at the trans Golgi [45, 46, 47]. Due to their possible role of sphingolipids in the LDs biogenesis, they have attracted a growing attention on recent years [45, 46, 47]. Sphingomyelin

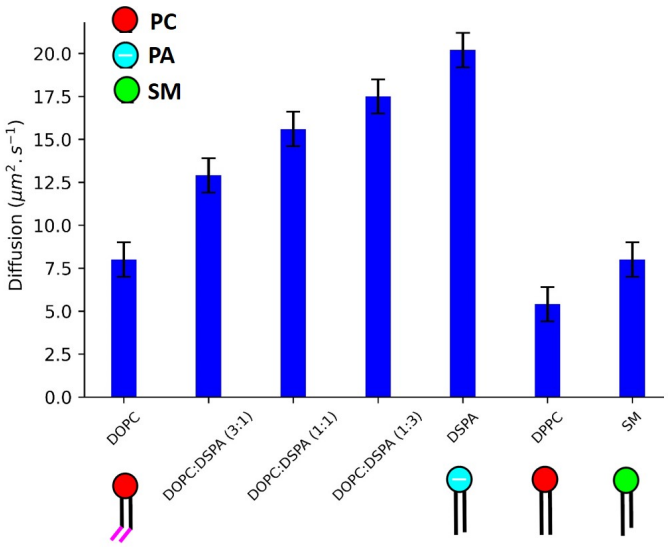

Figure 3: Diffusion constants D for unsaturated phospholipids (DSPA, DPPC, and SM) and mixtures of DOPC and DSPA at a triolein/buffer interphase extracted from FRAP experiments in comparison to values from DOPC. A schematic representation is provide for PC lipids (red head), SM (green head) and the charged PA (light blue head).

(SM) has a PC head with a fatty acid group and a tail that has a single saturation in contact with the head. Thus, we study this lipids with other saturated lipids. SM molecule has a geometry close to a cylindrical molecule. The relatively low diffusion constant of a pure SM monolayer of $D \approx 8\ \mu m^2 \cdot s^{-1}$, and the corresponding low surface tension $\gamma \approx 1.2$ mN/m, Fig.3, indicate that despite of the slightly conical shape of the SM, the associated lipid packing is dense, similar to an assembly of cylindrical molecules, in agreement with literature [38, 48].

Sterol, Sterol ester and Perilipin-2. Sterols like cholesterol (chol) and cholesteryl ester (chol ester) are also important components of LDs [7, 8]. However, these molecules differ from previous lipids, as they are unable to form a lipid bilayer. As example, chol-ester is not a surface active molecule, it is a component of the LD core and not of the LD surface. Nevertheless, these sterols are important lipids and it is interesting to investigate their influence on the phospolipids monoloayer properties. Besides, we also explore the effect of a LD protein (PLP-2) on the diffusion properties of phospholipids.

Cholesterol (Chol) is present in the ER membrane only in small amounts, namely below 6 % percent. Nevertheless, Chol may play a role in metabolic diseases that are involving LDs [4, 49]. Chol drastically alters the biophysical properties of the plasma membrane and thus, it is interesting to investigate the case of a lipid monolayer enriched with Chol. It is known that Chol increases both the bilayer tension and the bilayer fluidity, at least at low temperatures [50, 51, 52]. The measured surface tension for a DOPC monolayer with 10% Chol is ≈ 1.7 mN/m, while the surface tension of a DOPE monolayer with 10% Chol is ≈ 2.5 mN/m, i.e. the surface tension for both phospholipids are increased by about 10% compared to pure DOPC or DOPE monolayer, respectively, see table 1. The observed increase in surface tension indicates a lower packing density of lipids, which is expected to increase lipid diffusion. In fact, the measured diffusion constant for a DOPC monolayer with 10% Chol gives a value of $\approx 10\ \mu m^2 \cdot s^{-1}$, while that of a DOPE monolayer with 10% Chol gives $\approx 20\ \mu m^2 \cdot s^{-1}$, see Fig. 4, i.e. the diffusion constant also increased by about

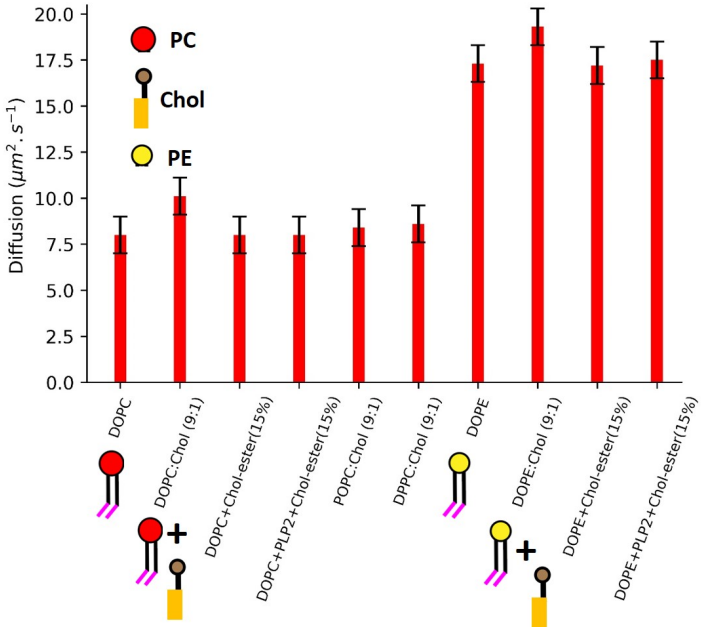

Figure 4: Diffusion constants D extracted from FRAP experiments for cholesterol containing DOPC, DOPE, POPC and DPPC monolayer at triolein-buffer interface, and DOPC and DOPE monolayer at cholesterol-ester containing triolein-buffer interface with and without PLP2. Schematic representation is provide for cholesterol and DOPC.

10% compared to pure DOPC or DOPE monolayer. This suggests that the increase in surface tension and lipid diffusion is indeed due to decreased lipid packing.

Cholesteryl ester, a dietary lipid, is an ester of cholesterol found in LDs [53]. This molecule differs from the previous molecules in that it is not localized at the surface of LDs but it is only present in the LD core. This is interesting because cholesterol ester appears to affect the recruitment of some LD proteins, such as PLP2, when they enrich the LD composition [30, 31]. The surface tension of a DOPC monolayer at the interface between triolein-buffer with 15% cholesteryl ester in triolein was measured to be $\gamma \approx 1.5$ mN/m, i.e. the same value as for triolein not containing cholesteryl ester. Similarly, we also measured the surface tension for a DOPE monolayer to be ≈ 2.3 mN/m no matter if the triolein contains 15% cholesteryl ester or not. The corresponding measured diffusion constant yields a value of $D \approx 8\mu\mathrm{m}^2{\cdot}\mathrm{s}^{-1}$ for a DOPC monolayer with 15% cholesteryl ester in triolein, and a diffusion constant $D \approx 17\mu m^2 \cdot s^{-1}$ for DOPE monolayer with 15% cholesteryl ester in triolein, see Fig. 4. From this, we can conclude that cholesteryl ester molecules remain in the triolein phase and hardly change the surface properties of the lipid decorated triolein-buffer interface. Similarly, we observe no measurable change in the surface tension of the phospholipid-decorated triolein-buffer interface or in the diffusion constant compared with a pure DOPC or a pure DOPE monolayer, respectively, when the aqueous buffer contains 1 μM of PLP2, regardless of whether the triolein phase contains 15% cholesteryl ester or not. Thus, either the lipid packing is hardly affected by the proteins or their concentration at the LD surface was not high enough to produce a notable effect, cf. Fig. 4.

3. Conclusion

We investigated the diffusion properties of lipids on the surface of model lipid droplets. For that, we produced a model lipid droplet interface made of a triolein-buffer interface in a microfluidic chamber that is decorated with a

Table 1: Table summarizing the measured surface tension values γ and diffusion constants D for triolein-buffer interfaces decorated with monolayers made of a single lipidic component. More results including lipid mixtures are available in Figs. 2,3 & 4.

Lipids	DOPC	DOPE	POPC	DPPC	DSPA	SM
γ (mN/m)	1.5±0.3	2.3±0.3	1.3±0.3	1.1±0.3	2.6±0.3	1.2±0.3
D (μm^2/s)	8.1±1	17.0±1	6.8±1	5.4 ±1	20.2±1	7.9 ±1

lipid monolayer of variable composition. FRAP experiments were conducted directly at this interface and the corresponding diffusion constant for the lipids was obtained as function of the molecular composition of the lipid monolayer. Based on geometrical (cylindrical, conical) and chemical (electrostatic charge, saturation) properties of the respective lipids, we argued that the lipid packing is the key parameter to understand the measured results. In this context, monolayers with high lipid packing have low lipid motility. And monolayers with low lipid packing have high lipid motility. This reasoning was supported by surface tension measurements as high lipid packing means low interfacial tension and low lipid packing means high interfacial tension. By extrapolation, this study provides an estimate of defect density on the LD surface for the lipids composition tested. This point is important because defects seem to play a key role in protein partitioning on LD surface [17].

4. Methods

Molecules. For the preparation of molecular monolayer that cover the model lipid droplets, i.e. the triolein-buffer interface, the following molecules were purchased from Avanti Polar Lipids: 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-distearoyl-sn-glycero-3-phosphate (sodium salt) (DSPA), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-(10Z-heptadecenoyl)-2-hydroxy-sn-glycero-3-phosphocholine (LPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-

3-phosphoethanolamine-N-methyl (N-DOPE), cholesterol (Chol), N-stearoyl-D-erythro-sphingosylphosphorylcholine (SM), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), Cholesteryl ester (Chol ester). Glyceryl trioleate (triolein) and squalene were purchased from Sigma-Aldrich. Recombinant human denaturtared perilipin 2 protein (PL2) was purchased from Abcam (ab181932). (FITC-DOPE) 1,2-dioleoyl-sn-glycerol-phospho-ethanolamine Fluorescein was purchased from ChemCruz (Santa Cruz Biotechnology).

Interfacial Tension Measurements. The interfacial tension of phospholipid decorated triolein-buffer interfaces was determined by the pendant drop method using a contact angle measurement device OCA 20 (DataPhysics, Germany). For this method, a transparent cuvette is filled with the lower density fluid, here the triolein-phospholipid mixture ($\rho_{oil} = 0.91$ g/cm^3) and a pendant drop containing a defined volume of the denser fluid, here the buffer solution ($\rho_{buffer} = 0.998$ g/cm^3), is produced from a hollow needle immersed in this liquid. While gravity drags the droplet down, buoyancy and surface forces keep the droplet in place. Based on a contour fit to the pendant droplet, the software SCA 20 extracts both size and shape of the droplet and automatically computes the corresponding interfacial tension. This procedure is most reliable when the droplet size is close to detachment from the needle, and we thus set the droplet volume to (2.0 ± 0.5) μl.

μChip Fabrication and Monolayer Formation. Using computer software (Autocad), we created a drawing in the form of a cylinder with 3 mm diameter, which was produced using a 3D printer. This cylinder was positioned in the center of a glass petri dish. After setting up the mold, mixed and degassed PDMS (Sylgard 184 – Dow Corning) was poured onto the mold with a resulting layer thickness of about 3 mm so that the cylinder is not fully immersed in the PDMS. After curing the PDMS at 60 °C for 6-7 hours, the cylinder was removed and square pieces with the cylindrical hole in the center were cut from the PDMS and removed from the petri dish. The final device was fabricated by attaching the square PDMS piece to a PDMS coated glass cover slip (Sylgard 184 spin

coated for 5 min at 3000 rpm) using plasma bonding (Diener electronics). The thus fabricated device was placed it on a hot plate at 60 °C for 1 hour to achieve higher bonding strength.

To produce the triolein-lipid mixture, a specific amount of lipid was dissolved in Ethanol and mixed with 8% DOPE-FITC (dissolved in chloroform). This solution was then dried under vacuum. Then triolein was added to the dried components and mixed in an ultrasonic bath.

To form the monolayer, 6 μl of the as-prepared triolein-lipid mixture was first placed in the cylindrical opening of the PDMS micro chip, and then 20 μl of 150 M KCl buffer was added on top. Within seconds, a phospholipid monolayer forms at the triolein-buffer interface, with a composition similar to the lipid composition of the originally prepared triolein solution.

FRAP Experiments. The mobility of phospholipid monolayers at triolein-buffer interfaces were determined by fluorescence recovery after photobleaching (FRAP) measurements using an inverted Zeiss microscope, Axio Observer Z7. FITC-DOPE (495 nm/515 nm) is used as a dye for our FRAP experiments due to its very low photostability. For the FRAP measurements, a pinhole (diameter of 150 μm - Edmund Optics) was placed between the microscope tray and the bottom of the microfluidic device. Once the lipid monolayer is formed, the microscope is focused on the center of the monolayer using a 40× objective and blue LED light (469/38 nm) is emitted continuously for 30 to 60 s at maximum power (zeiss colibri 7) to bleach a spot with a diameter of about 150 μm. Subsequently, the pinhole is removed and the 40× objective is changed to a 10× objective and fluorescence recovery is recorded with a frame rate of 1 s using the same blue wavelength excitation but now with the lowest illumination power ($\approx 1\%$). The fluorescence recovery, i.e. the fluorescent intensity of the bleached area was extracted automatically using the software ImageJ. The diffusion constants were obtained via a Soumpasis fit [32] to the intensity data using Origin8 (OriginLab). For information, we choose to performed a maximum of 2 FRAP experiments by phospholipid monolayer. After two measurements, we clean the

chip (ethanol, acetone) and replace the chemical to form a fresh triolein-buffer interface.

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