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Structure of lipid multilayers via drop casting of aqueous liposome dispersions

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Abstract Understanding the structure of solid supported lipid multilayers is crucial to their application as a platform for novel materials. Conventionally, they are prepared from drop casting or spin coating of lipids dissolved in organic solvents, and lipid multilayers prepared from aqueous media and their structual characterisation have not been previously reported, due to extremely low lipid solubility (i.e. ~10^{-9} M) in water. Here, using X-ray reflectivity (XRR) faciliated by a "bending mica" method, we have studied the structural characteristics of dioleoylphosphatidylcholine (DOPC) multilayers prepared via drop casting aqueous small unilamellar and multilamellar vesicle or liposome (i.e. SUV and MLV) dispersions on different surfaces, including mica, positively charged polyethylenimine (PEI) coated mica, and stearic trimethylammonium iodide (STAI) coated mica which exposes a monolayer of hydrocarbon tails. We suggest that DOPC liposomes served both as a delivery matrix where an appreciable lipid concentration in water (~25 mg mL^{-1} or 14 mM) was feasible, and as a structural precursor where the lamellar structure was readily retained on rupture of the vesicles at the solid surface upon solvent evaporation to facilitate rapid multilayer formation. We find that multilayers on mica from MLVs exhibited polymorphism, whereas the SUV multilayers were well ordered and showed stronger stability against water. The influence of the subtrate chemistry (i.e. polymer coating, charge and hydropobicity) on the multilayer structure is discussed in terms of lipid-substrate molecular interactions determining the bilayer packing proximal to the solid-liquid interface, which then had a templating effect on the structure of the bilayers distal from the interface, resulting in the overall different multilayer structural characteristics on different substrates. Such fundamental understanding of the correlation between the physical parameters that characterise liposomes and substrate chemistry, and the structure of lipid multilayers, underpins potential development of a simple method via an aqueous liposome dispersion route for inclusion of hydrophilic functional additives (e.g. drugs or nanoparticles) into lipid multilayer based hybrid materials, where tailored structual characteristics are an important consideration.

1. Introduction

Lipid bilayers have been widely studied since Mueller's description of the classic black lipid membrane (BLM) in 1960s[1] and Tamm and McConnell's report on supported lipid bilayers (SLBs) in 1980s[2]. Using SLBs as model membranes[3], biological processes at the cellular level, such as viral attack[4, 5], cellular signalling events[6, 7] and ligand-receptor interactions[8-11] have been investigated. In addition, SLBs have found themselves in many applications, *e.g.* microcontact printing[12, 13] and photolitography[14] to produce biofunctional nanomaterials such as label free biosensors, and several reviews exist on these topics[15-17].

Using lipid bilayers as building units, lipid multilayers have also been prepared in which tens to thousands of bilayers can be stacked forming ordered structures. Phosphatidylcholine lipids have been widely used, as they are the main component of cell membranes. Various applications using lipid multilayers have been developed mainly in biology[18-20] and nanotechnology[21, 22], as model membranes to study interactions with drugs and nanoparticles, and also as a platform for functional materials. For example, dioleoylphosphatidylcholine (DOPC) lipid multilayers with thickness between 5–100 nm on silicon wafers, glass, evaporated metal films, polystyrene[22] and polymethyl methacrylate (PMMA)[21] substrates have been prepared by dip-pen nanolitography as photonic components. Similarly, DOPC, dioleoylphosphatidylethanolamine (DOPE), and dipalmitoylphosphatidylcholine (DPPC) gratings have been prepared on polydimethylsiloxane (PDMS) and glass, as potential biosensors for detection of lipid-

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protein and lipid-drug interactions, and as a starting point for microarray development[18-20]. Lipid multilayers incorporating cholesterol- or sphingomyelin-enriched lipid multilayers on silica substrates have also been prepared for potential application in novel membrane-based functional materials and devices[23].

Many studies have thus aimed to optimise lipid multilayer formation for enhanced structural order and stability. Whilst the most common method to prepare SLBs is to rupture liposomes onto a substrate, the preparation of lipid multilayers on solid surfaces involves drop casting[24-30] or spin coating[31, 32] from an organic solution of dissolved lipids (forming samples typically with thousands or tens of bilayers, respectively for the drop- and spin-casting methods). In a series of studies, Salditt *et al*.[25, 28, 29, 31-35] reported the formation of lipid multilayers by spin coating a lipid solution in an organic solvent onto silicon and glass substrates, showing that the number of stacked bilayers could be controlled by the volume and concentration of the solution and the spin speed. Dimyristoylphosphatidylcholine (DMPC) multilayers on silica were investigated by (X-ray reflectivity) XRR[31], neutron reflectivity (NR)[32, 34], atomic force microscopy (AFM), and optical microscopy[32]. XRR and NR profiles collected at temperature above the lipid melting temperature, T_g, revealed single crystalline membranes with the stacking of at least 10 bilayers ~5 nm in thickness. This correlated well with the step size of surface features revealed by AFM imaging. However, no features were observed with optical microscopy.

Tristan-Nagle[36] reported a "rock and roll" method to prepare multilayers, in which a lipid solvent solution was deposited on the substrate fixed on a vial that was rocked and rolled manually under controlled conditions. Different substrates (*i.e.* glass, mica, and silicon wafer) and lipids (*i.e.* phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE) with different chain lengths) were investigated. AFM imaging suggested that the multilayers were more ordered than those obtained by spin coating. Similarly to the drop casting method, the "rock and roll" method produced multilayers comprising hundreds to thousands of bilayers.

A main drawback for lipid multilayers is their low stability, as they are unstable in water-vapour atmosphere[32], and can delaminate underwater thus losing their structural order[37]. Hydration of thick DOPC and dioleoyltrimetylammoniumpropane (DOTAP) multilayers (~1200 bilayers) prepared by drop casting on silicon wafer was found to lead to an increase of the lamellar spacing, *d*, as observed by electron density X-ray diffraction (EDXD)[24]. Similar results were observed by Cavalcanti *et al*.[26], when anticancer drugs were intercalated in lipid multilayers formed by DPPC and DOTAP. Multilayer unbinding can also be caused by heating[29] or by applying an electric field[25] to the lipid multilayer.

So far, all the lipid multilayer studies discussed have used organic solvents as the medium, as lipids dissolve in them readily at high concentrations (*e.g.* with concentrations of 2–20 mg mL^{-1} typically used), and the solvent can be easily evaporated after deposition. Dissolving a mixture of different lipids in an organic solvent also affords the possibility for the formation of mixed lipid multilayers[27]. It would be desirable, however, to develop an alternative route from an aqueous lipid dispersion, *e.g.* to facilitate a pathway for inclusion of hydrophilic additives. However, lipid solubility in water is extremely low (with the solubility of biological lipids in the order of ~nM), making the use of water as a solvent less straightforward, with very few related studies in the literature.

In the present work, DOPC lipid multilayers have been prepared by drop casting an aqueous liposome dispersion on different surfaces: negatively charged mica, positively charged PEI-coated mica, and STAI-coated mica, which exposes a monolayer of hydrocarbon tails making it more hydrophobic. Use of DOPC liposomes afforded an appreciable concentration of the lipid dispersion in water (~25 mg mL^{-1}), and also served as a structural precursor for subsequent multilayer formation during droplet evaporation. XRR was used to characterise the structure of the lipid multilayers, facilitated by a "*bending mica*" method[38, 39, 40]. Such XRR results on mica are unprecedented (and we note that neutron reflectivity has also been recently successfully applied to the mica surface[41]). The structures of multilayers obtained from dispersions of small unilamellar vesicles (SUVs) and multilamellar vesicles (MLVs) with different liposome sizes were compared. Polymorphism was observed in some samples, indicating the coexistence of bilayer domains with different *d*-spacing. The influence of different substrate chemistry on the multilayer structure is discussed in terms of lipid-substrate molecular interactions affecting the bilayer packing at the interface. Our results on the structural characteristics of the DOPC multilayers via drop casting an aqueous liposome dispersion, and their correlation with the physical parameters that characterise liposomes and substrate chemistry, are of fundamental relevance and also offer a potential route to facilitate incorporation of hydrophilic actives (*e.g.* drugs and nanoparticles) in lipid multilayer based hybrid materials.

2. Materials and Methods

2.1. Materials and sample preparation

DOPC lipid (>99% purity, MW = 786.113 g mol^{-1}) in chloroform (25 mg mL^{-1}) was purchased from Avanti® Polar Lipids, Alabaster, Alabama, and poly(ethyleneimine) (PEI; formula $(CH_2CH_2NH)_x$, MW = 13,000 g mol^{-1}) from Polymer Source™, Quebec, Canada, both used with no further purification. Its main solid-ordered (SO) to liquid-disordered (LD) transition temperature is T_m = –17 °C, so that the lipid was in its LD phase at the temperature of the experiments. Surfactant stearic trimethylammonium iodide (STAI), known also as octadecyltrimethylammonium iodide (formula $CH_3(CH_2)_{17}N^+(CH_3)_3I^-$), was prepared from octadecyltrimethylammonium chloride (STAC) as described in Ref. [42] and also outlined in the Electronic Supplementary Information (ESI) section. Natural muscovite mica ($KAl_2(Si_3Al)O_{10}(OH)_2$) of A1 special grade was purchased from SJ Trading®, New York. Ultrapure Milli-Q® water with resistivity of 18.2 MΩ cm^{-1} and a total organic content (ToC) of 3-4 ppb at 25 °C, chloroform (VWR, (≥99.8% purity) and nitrogen (Air Liquide, oxygen free) were used for sample preparation and XRR measurements.

Mica surfaces were prepared, first by cutting an original sheet (of size ~25 cm × 15 cm × 1 mm) with a pair of precision scissors, and then by hand-cleaving, into smaller pieces. They were further cut into pieces of 3 × 1 cm in size and ~300 µm in thickness, as required for the XRR liquid cell[39, 43]. STAI coated mica (*cf*. ESI) was prepared by immersing a freshly cleaved mica piece into a 1 mg mL^{-1} aqueous STAI solution at 70 °C for 30 s, and this was followed by immersion in Milli-Q water at 70 °C for 30 s. The contact angle of a water droplet on such STAI-coated mica was ~71° (ESI), indicating that it was more hydrophobic than bare mica. PEI-coated mica was prepared by dipping a freshly cleaved mica piece into a 100 ppm polymer solution for 10 min at room temperature (RT). The STAI- and PEI-coated surfaces were then left to dry in a laminar flow hood overnight, and all the surfaces were then kept in sealed glass vials to avoid any contamination, with the mica pieces positioned in such a way that only their bottom edge and top corners came into contact with the glass vial.

2.2. Preparation of lipid multilayers

A designated amount of DOPC in $CHCl_3$ (25 mg mL^{-1}) was weighed into a 7 mL glass vial, and the solvent was removed with a gentle N_2 stream to obtain a dried uniform crystal-free lipid film. The lipid film was first hydrated with Milli-Q water to a concentration of 14 mM and sonicated for ½ hour (h) at RT (above the DOPC melting temperature (T_m) of –17 °C). The obtained dispersion was gently shaken manually and then the size of the resultant liposomes was checked with dynamic light scattering (DLS), using a Malvern Nano Zetasiser ZS (Malvern Instruments, Malvern, Worcestershire, UK). DLS measurements revealed that a multilamellar vesicle (MLV) dispersion was obtained, containing a mixture of MLVs of ~150 nm in diameter, and bigger aggregates typically of size ~300-1500 nm, with a polydispersity index (PDI) ~1. These results are consistent with previous reports[44, 45].

The MLV dispersion was then extruded through first 0.4 µm and then 0.1 µm pore size polycarbonate membranes (Avanti® Polar Lipids Inc., Alabaster, Alabama) 3 times each using a LIPEX™ 10 mL Thermobarrel Extruder (Northern Lipids Inc., Burnaby, Canada) under ~20 bar pressure of N_2 or 21 times each using a manual Avanti® Mini Extruder apparatus (Avanti® Polar Lipids Inc., Alabaster, Alabama). DLS measurements of the obtained dispersion showed that single lamellar vesicles or liposomes (SUVs) of diameter ~90 nm with a narrow size distribution (PDI = 0.05) were formed. For drop casting, both the SUV and MLV DOPC dispersions were diluted to 2 mg mL^{-1} with Milli-Q water, and 100 µL of each dispersion was dropped onto the substrate, with care taken to ensure that the droplet formed did not spill over the mica sheet. The samples were left to dry in a vacuum oven at RT for 2 h to ensure the removal of water, with a dried footprint of the thin film ~1 cm^2 in area and ~2 µm in thickness. The multilayers thus formed were kept in clean sealed vials at 4 °C until synchrotron X-ray reflectivity measurements were performed.

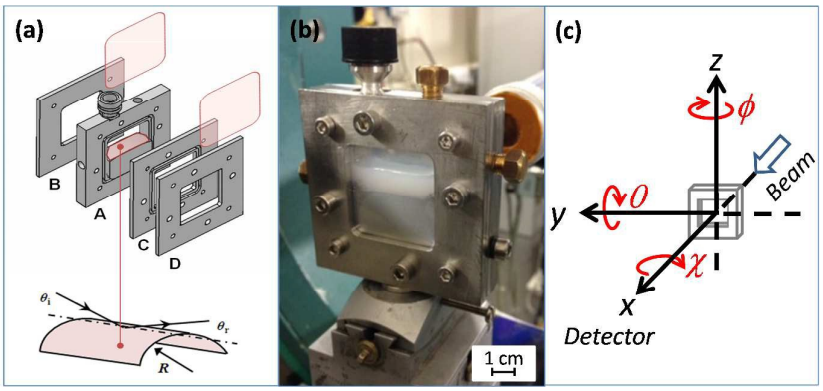

Figure 1: (a) Schematic representation of the main components of the XRR liquid cell, consisting of four stainless steel plates (A-D) and a cylindrical stage on which a 3 × 1 cm mica is gently bent. XRR measurements are made along the rigidified bending axis. (b) A photograph of the XRR liquid cell as mounted on the goniometer at ESRF beamline BM28. The white/yellow scale bar in (b) is 1 cm. (c) translational (*x*, *y*, and *z*) and rotational (θ, ϕ, and χ) axis of the Huber diffractometer with respect to the XRR cell (grey, schematic), beam, and detector. (*cf* ESI for

2.3. X-ray reflectivity (XRR) liquid cell and Synchrotron XRR measurements

The liquid cell used in the XRR measurements (Figure 1) employs a "bending mica" method[40], with a mica piece gently bent over an underlying cylindrical sample support of radius *R* = 7.5 cm and clamped *via* two small plates. This enhances the rigidity of mica along the bending axis, thus providing sufficient flatness for the XRR

measurements along this axis. The liquid cell was described in detail elsewhere[43] (see ESI for a brief description of the liquid cell and sample alignment).

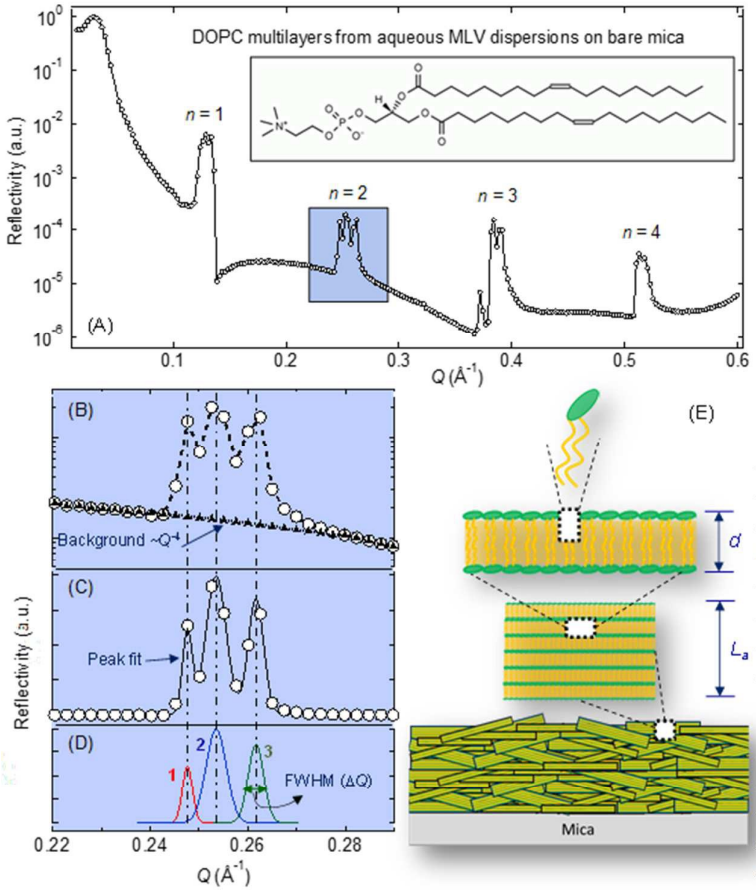

Figure 2: (A) Experimental XRR curve of DOPC multilayer from an MLV dispersion on bare mica, collected in air at room temperature. (B) An enlarged view of the reflectivity on a log-linear scale around the second order (n = 2) Bragg peak (as enclosed in the rectangle in (A)), with the background fitted to Q^{-4}; (C) The residual peak after background subtraction (circles), and its fit (black curve) calculated with the IGOR Pro "Multipeak Fitting" operation; (D) The fitted peak could be decomposed into three Gaussian peaks (*cf.* Table 1), indicating polymorphism in the thin film. (E) Schematic depiction of a multilayer thin film, comprising stacked DOPC lamellar domains with the lattice plane of the bilayer (of spacing d) approximately parallel to the substrate. The lower limit of the domain size perpendicular to the lattice plane is indicated by the coherence length (L_a), obtained from analysis of the broadening (as defined by the FWHM ΔQ) of the peaks in (D) using the Scherrer equation.

XRR measurements were performed at beamline BM28 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France, and also at beamline I07 at the Diamond Light Source (DLS), Didcot, UK. XRR measurements were typically made at room temperature for all the samples in air, then water was injected into the liquid cell and the measurement repeated, with an integration time of ~1–5 seconds at each angle (*e.g.* in the range θ_i = 0.06°–2.6° corresponding to a Q range of ~0.015 to 0.64 Å^{-1} for λ = 0.886 Å at BM28) at a typical step size of 0.01°, where the momentum transfer vector normal to the sample surface is $Q = (4\pi\sin\theta_i)/\lambda$. The specularly reflected intensity was detected at each angle $\theta_r = 2\theta_i$ by an avalanche photodiode detector (APD) for at ESRF BM28, and by a Pilatus 100K 2D detector at Diamond Light Source I07. The resulting reflectivity curve can be plotted as reflectivity (a.u.) versus Q (Å^{-1}). Measurement details and sample alignment procedures are given in ESI.

3. Results and Discussion

Figure 2A shows an example XRR curve from a thin film sample drop cast from a DOPC MLV dispersion on bare mica. A distinct feature in the XRR curves for the DOPC multilayer thin films in air is the presence of sharp Bragg peaks up to the 4th order (n = 4) of diffraction (also Figure 3A for an SUV sample), as compared to that of a bare mica (*cf.* Figure 5F). These peaks are attributed to the diffractions from the lattice planes of the highly ordered multilayers approximately parallel to the substrate, and the ratio of 1:2:3… in the peak Q_n positions (of order n = 1, 2, 3…) is consistent with a lamellar structure. The d-spacing, which is the lipid bilayer thickness or the periodic distance between the lamellar lattice spacing, can be calculated as

$$d = \frac{2n\pi}{Q_n}, \quad (1)$$

yielding d ~ 46.8–50.6 Å (±0.2 Å) (Table 1; MLVs), which is consistent with a DOPC bilayer thickness[24]. The error analysis details are given in ESI. The overall shape of the curve results from the morphological surface characteristics (*i.e.* coverage, relaxation of the upper layer, roughness), and a detailed structural model incorporating these features is presented

Table 1: Peaks Q position, correspondent thickness d, and coherence length L_a for the DOPC multilayers from aqueous dispersions of MLVs. The polymorphism of MLV multilayers is evident from the presence of multiple peaks (denoted as $Q_{n,(1\text{-}4)}$) in the same order (n), and up to four d-spacing values were registered, albeit not all the constituent peaks could be resolved for the Bragg peaks due to insufficient angular resolution. Errors from the fittings are reported when they are greater than 0.5% for the Q values and greater than 0.1 Å for the d spacing values. [a.] Large uncertainties due to small number of fitted data points for the resolved peaks.

DOPC Dispersion MLVs				
Parameter	Diffraction order n (*cf.* Figure 2)			
	1	2	3	4
$Q_{n,1}$ (Å^{-1})	0.125	0.247 ± 0.003	0.372	
d (Å)	50.1	50.9 ± 0.6	50.7 ± 0.1	
L_a (Å)	808.9 ± 50	3271.8 ± ~1000[a]	2408.6 ± 236.1	
$Q_{n,2}$ (Å^{-1})		0.253	0.383	0.511
d (Å)		49.6	49.2	49.1
L_a (Å)		1258.9 ± 33.5	1655.0 ± 49.3	2539.0 ± ~1000[a]
$Q_{n,3}$ (Å^{-1})	0.130	0.262	0.390	0.516
d (Å)	48.2	48.0	48.3	48.7
L_a (Å)	1199.4 ± 50	1676.6 ± 49.3	1348.1 ± 50.2	753.6 ± 38.1
$Q_{n,4}$ (Å^{-1})	0.135			
d (Å)	46.6			
L_a (Å)	2370.8			

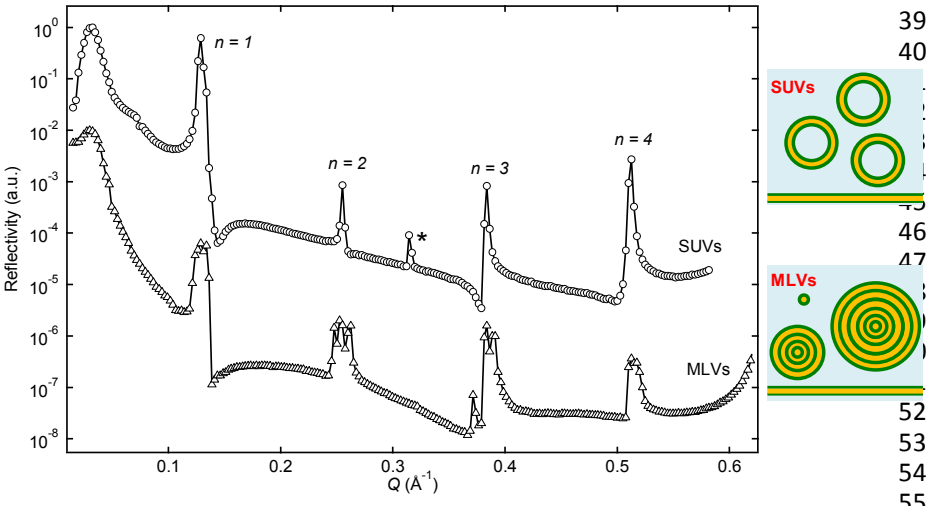

Figure 3: XRR curves for DOPC multilayers on bare mica from an SUV (circles, top curve) and an MLV (triangles, bottom curve) dispersion. The peak (marked with *) at $Q \sim 0.32$ Å is the mica forbidden half Bragg peak.

elsewhere[46]. At Q = 0.6 Å^{-1} the reflectivity from all the samples starts to increase, a feature characteristic for XRR curves on mica due to the presence of mica's Bragg peak (Q = 0.64 Å^{-1}). Mica's forbidden half Bragg peak (Q = 0.32 Å^{-1}) is also present in some of the curves (always indicated with an asterisk (*)), which is due to mica's monoclinic unit cell encompassing two lattice layers.

Within a small Q range around the Bragg peaks, multiple peaks could be resolved, particularly pronounced for the n = 2, 3 peaks, revealing a complex structure of the sample. Here we focus on the analysis of the Bragg peaks using the Scherrer equation to yield the coherence length L_a, the physical meaning for which is the lower limit of the crystalline domain size perpendicular to the mica surface; it thus can be used as an indication of the structural order of the multilayer[47, 48]. The analysis was performed in IGOR Pro as follows. As an example, for the 2nd order peak (n = 2) in Figure 2A, the reflectivity data in the Q range (0.22-0.29 Å^{-1}) enclosing the peak was selected and the background reflectivity was fitted to a 4th order polynomial (*i.e.* $\sim Q^{-4}$; dotted black curve in Figure 2B), and subtracted from the reflectivity data. The residual peak (circles in Figure 2C) was subsequently fitted with the IGOR Pro "Multipeak Fitting" operation (black curve in Figure 2C), which could be decomposed into three Gaussian profiles (Figure 2D). As listed in Table 1, the peak Q positions for the three peaks (denoted as $Q_{n,(1\text{-}3)}$ for MLVs (n = 2); $Q_{2,1}$ = 0.247 Å^{-1}, $Q_{2,2}$ = 0.253 Å^{-1}, and $Q_{2,3}$ = 0.262 Å^{-1}) correspond to three d-spacing values (50.9 (± 0.6), 49.6 and 48.0 Å respectively; for the last two d values the error from the fitting is less than 0.01 Å). Up to four d values were observed due to polymorphism, although not all the corresponding peaks could be resolved at each Bragg peak (*cf.* Table 1). At small Q the peaks overlap more, making it more difficult to resolve them. On the other hand, at very high Q the reflectivity is considerably diminished (as it scales $\sim Q^{-4}$), and this again could limit the resolution in de-convoluting the multiple peaks. Such a difference in d could be due to different lipid packing, arising from when the MLVs and other lipid aggregates ruptured to form multilayers. It could also be due to a slightly different residual water content in different domains, as the d-spacing of a DOPC multilayer has been observed to vary with the variation of humidity[49].

The full width half maximum (Figure 2 (D); FWHM, ΔQ) values were also obtained from the analysis. The crystalline domain size (or coherent length, L_a, listed in Table 1) along the direction normal to the surface is inversely proportional to ΔQ and can be calculated using Scherrer's equation:[50]

$$L_a = \frac{2\pi K}{\Delta Q}, \quad (2)$$

where K is the Scherrer constant, a shape factor ~1 (*e.g.* a common value is 0.94 for a cubic symmetry but it varies depending on the shape of the crystal and on how the width is determined). Then the number of bilayers m in the domain could be estimated as $m = L_a/d$.

In Figure 3, XRR curves of the multilayers on mica obtained from drop casting of SUV and MLV dispersions are compared. In contrast to the MLV sample above, the Bragg peaks from the SUV sample are well defined single peaks, indicating that the multilayers were largely monomorphic with a constant d of 49.1 Å. The d values

Table 2: The bilayer thickness d, peak position Q_n, coherence length L_a, and the corresponding number of layer in the domain (m) for DOPC multilayers obtained from SUVs bare mica in air. Errors for d and Q_n are < 0.05 % from the fitting.

DOPC Dispersion	SUVs			
Diffraction Order n	Q_n (Å^{-1})	d (Å)	L_a (Å)	No. layers m
1	0.128	49.1	1638.6 ± 31.8	34
2	0.255	49.3	2308.3 ± 26.4	47
3	0.384	49.1	2107.6 ± 33.0	43
4	0.512	49.1	1935.3 ± 17.6	39

Table 3: The bilayer thickness d, coherence length L_a, and the corresponding number of layer in the domain m for DOPC multilayers obtained from SUVs and MLVs on bare mica, in air and in water. Errors for d are < 0.1 % from the fitting. a. Average values: for SUVs from all diffraction orders; for MLVs from the 2nd and 3rd resolved peaks in all diffraction orders (where present in air).

Condition	DOPC liposome dispersion	d (Å)	L_a (Å)[a]	No. layers m
In air	SUVs	49.1	1997.4 ± 27.2	41
	MLVs	49.0	1435.8 ± 52.2	31
		48.3	1291.6 ± 62.5	26
Under water	SUVs	62.6	1078.3 ± 44.1	17
	MLVs	No Bragg peaks observed		

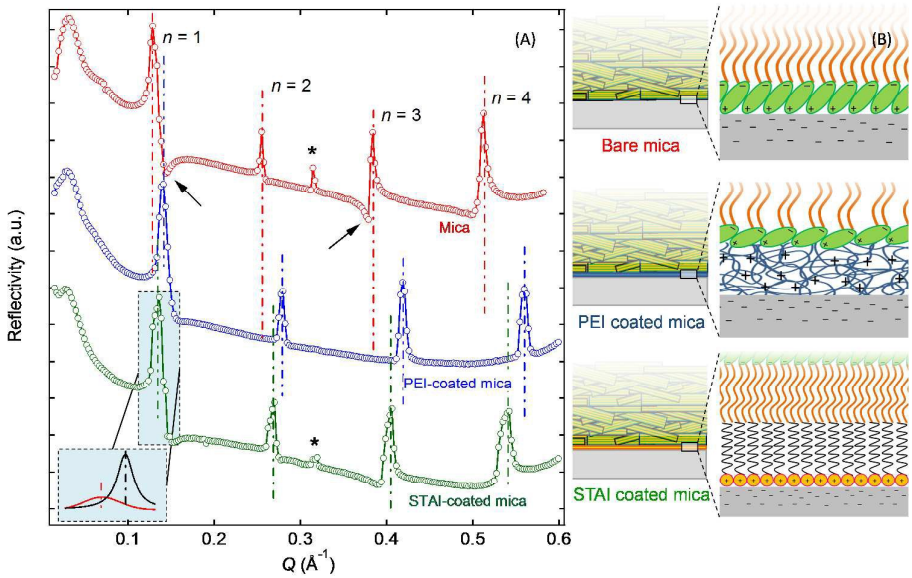

Figure 4: (A) XRR curves for DOPC on bare mica, PEI-coated mica, and STAI coated mica collected in air at room temperature. The forbidden mica half Bragg peaks are indicated by *. The arrows indicate the presence of negative peaks, attributed to the relaxation of the top lipid layer at the air-film interface. The light blue rectangle shows a magnification of the fitting of the first Bragg peaks for the STAI-coated mica sample. (B) Schematic representation showing the three different substrates.

calculated from all the four orders of the Bragg peaks (*cf.* Table 2) are also in close agreement (the errors associated with *d* from each peak are smaller than 0.01 Å, and the average deviation from the mean value is 0.1 Å), further indicating well defined bilayer structures in the multilayer lamellae. In Table 3, the DOPC multilayer characteristics obtained for SUVs and MLVs samples are compared. The average domain size for the SUV sample in air (L_a = 1997.4 ± 27.2 Å) is much greater than that of the MLV sample (L_a = 1339.7 ± 59.0 Å, calculated as the average of the third peak of each diffraction order), and since the lattice spacing for the two samples is almost the same, it confirms a more ordered structure in the case of SUVs, the number of bilayers in the domain being ~41 for SUVs while only ~26 for MLVs. It is conceivable that monodispersed SUVs provided a more uniform structural template upon rupture, leading to a more organised structure; whereas the MLV dispersion consisted of a plethora of aggregates in the size range ~150–1500 nm, as revealed by DLS, which frustrated packing upon rupture at the interface, leading to both the polymorphic and less ordered structure observed.

Figure 4(A) indicates that drop cast DOPC multilayers formed on all the substrates. In the case of PEI-coated mica, the Bragg peaks are slightly broadened (L_a = 1323.4 ± 22.1 Å) compared to that of the bare mica sample (L_a = 1997.4 ± 27.2 Å), suggesting a slightly less ordered structure. The peaks are also shifted towards higher *Q* values, pointing to a slightly thinner bilayer (by ~3 Å), as compared to the multilayers on bare mica. All the samples were measured under the same ambient conditions, so it is unlikely that the structural differences were due to different relative humidity, and consequently different hydration, levels. PEI and mica would have exhibited different surface charge densities, and the interfacial roughness on PEI-coated mica is expected to be slightly higher. We thus attribute the observed structural differences to the interactions between the DOPC headgroups and the PEI layer. The dipole of the zwitterionic headgroup is oriented with the negative charge proximal, and the positive charge distal, to the tail. The outermost positive charge enables PC-lipids to attach to mica and to negatively charged polymers. In the case of the positively charged PEI-coated mica, the headgroups must adopt an orientation and arrangement different from that on bare mica (schematically shown in Figure 4(B)), and this would consequently result in different lipid packing in the bilayer immediately adjacent to the surface. A thinner bilayer suggests that either tilting or interdigitation of the lipid tails occurred[39], due to a slightly relaxed packing. It is curious and interesting that the Bragg peaks from the PEI-coated mica sample are monomorphic, which indicates that the surface bilayer had a templating effect and the bilayer packing was largely retained throughout the thin film.

In the case of more hydrophobic STAI-coated mica as the substrate, the DOPC multilayer thin film exhibited polymorphism, for instance with the two decomposed peaks from the *n* = 1 Bragg peak shown in the inset at the bottom left corner of Figure 4, with corresponding *d*-spacing and coherence length values of (*d* = 47.8 Å, L_a = 747.1 Å) and (*d* = 46.1 Å, L_a = 1603.6 Å) respectively (the errors in the thicknesses *d*, and full width at half maximum ΔQ, from the fitting is ~ 0 Å, thus the error on L_a is also almost null). That is, the film constituted domains with (at least) two different lattice spacing, and those with slightly thinner bilayers were more ordered. It

is conceivable that, upon encountering the hydrophobic STAI monolayer, the DOPC SUVs would rupture and also need to undergo cleavage so that the hydrophobic lipid tails are unzipped from the liposome bilayers, facing towards and in intimate contact with the STAI layer. The packing density of the tails would be influenced by that of the STAI monolayer and also the STAI surface coverage. Subsequent surface templating effects would lead to such bilayer structures being largely retained throughout the film.

A noticeable feature in the XRR curves of the SUV DOPC multilayer thin film on bare mica is the "negative" peaks, as indicated by two arrows in Figure 4. As a detailed structural model shows,[51] this is due to the relaxation of the terminating monolayer at the air-film interface, most pronounced on bare mica and much less so on more hydrophobic STAI-coated mica and absent on PEI-coated mica.

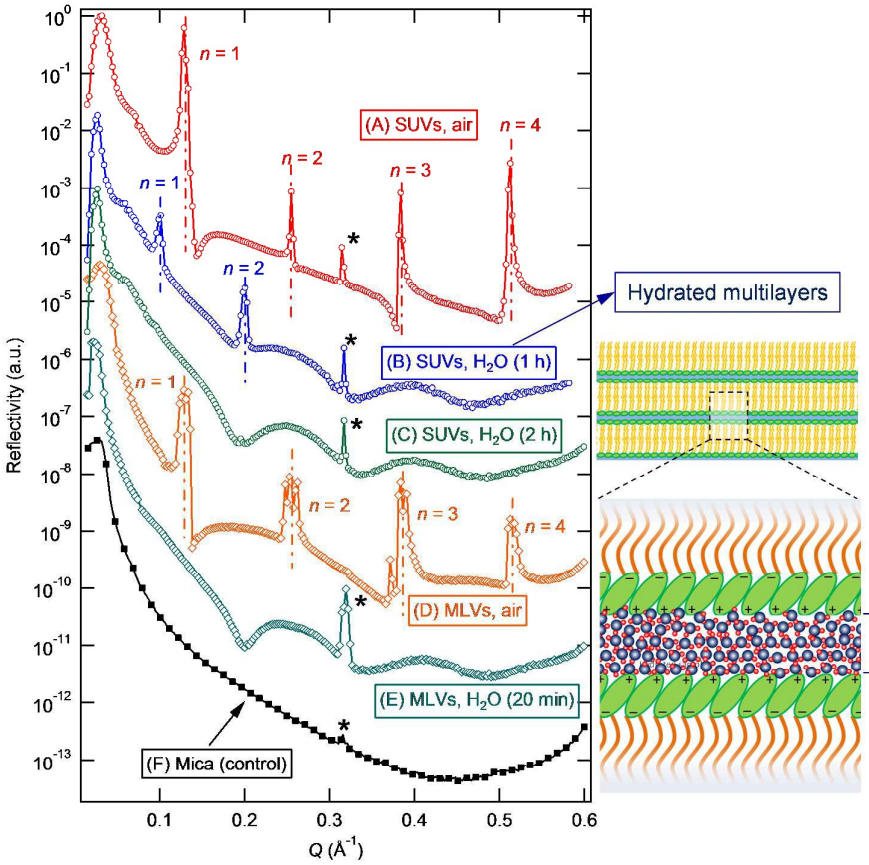

Figure 5: XRR curves of SUV DOPC multilayers on mica in air (A), in water after 1 h (B) and 2 h immersion (C). XRR curves of MLV DOPC multilayers on mica in air (D), and in water after 20 min immersion (E). For comparison, the XRR curve of a control bare mice piece in air (*i.e.* without any DOPC layers) is shown in (F). The hydrated multilayers in the SUV sample under water (Curve B) are shown schematically on the right hand side, with a hydration layer of thickness Δd ~ 13.5 Å per bilayer, corresponding to 4-5 molecular water layers.

It is known that lipid membranes are not stable under water[52] and bilayers tend to delaminate from lipid membranes[32, 37]. It is important to understand the structural transition of lipid multilayers under water, a rapid process difficult to probe experimentally. Relatively fast XRR scans due to the high synchrotron X-ray flux have allowed us to compare the XRR curves of the DOPC multilayers from SUVs and MLVs at the initial stage of water submersion (Figure 5). After 1 h under water, the Bragg peaks from the SUV DOPC multilayers shifted towards lower Q (Figure 5B), consistent with thicker bilayers (d = 62.6 Å) due to hydration of the headgroups. As compared to d = 49.1 Å in the ambient air, this represents bilayer swelling of Δd ~ 13.5 Å, corresponding to a hydration layer with 4-5 molecular water layers per bilayer. Similar swelling behaviour due to hydration has also been previously observed for supported purple membranes[53, 54] using XRR. Furthermore, the Bragg peaks of hydrated samples are broader and less intense than those of dry multilayers, with an L_a = 1078.3 ± 44.1 Å as compared to L_a = 1997.4 ± 27.2 Å in air (*cf.* Table 3), and the 3rd and 4th Bragg peaks are also absent. The loss of crystalline long-range order is due to lattice defects and bending fluctuations in addition to water-induced bilayer undulations, which would also give rise to variations in the bilayer thickness. However, no significant peak splitting (*i.e.* polymorphism) was observed. These observations suggest that bilayer hydration occurred soon after water addition, and the swelling of the bilayers was largely uniform, with water molecules permeating through the multilayer structure. This resulted in swollen bilayers, with an overall less ordered multilayer structure. Our results from a separate experiment (Figure S2 in ESI) show that the DOPC multilayer structure could be retained up to ~2 h in water, where similar bilayer swelling was also observed.

Prolonged water submersion led to the loss of the multilayer structure, evident from the disappearance of the Bragg peaks (Figure 5C; 2 h in water in this particular experiment). Instead, mild reflectivity oscillations called Kiessig fringes appear due to a lipid bilayer of thickness ~45.3 Å remaining at the mica-water interface[39].

Table 4: Bilayer thickness d, coherence length L_a (from n = 1 Bragg peak), and the minimum number of bilayer m in the lamellae domain for SUV DOPC multilayers on bare, PEI- and STAI-coated mica. For comparison, the L_a and m values obtained from the paracrystalline disorder analysis are also reported. Errors for d are not reported as they are almost null (*i.e.* at least < 0.05 %) from the fitting. [a]Values correspond to the two decomposed peaks (*cf.* Figure 4A). [b.] Multiple peaks are present for each Bragg peak due to polymorphism but not all resolved at each Bragg peak; here the third peak ($Q_{n,3}$) of the decomposed peaks from n = 1, 2, 3, and 4 Bragg peaks in the XRR curve for the MLV DOPC multilayer sample is used, which has also allowed the paracrystalline disorder parameter g (Figure 6) to be extracted.

Substrate for SUV DOPC multilayer	d (Å)	L_a (Å)	No. layers m	Paracrystalline disorder parameter g	L_a (Å) from Fig.6 and Eq.(4)	No. Layers m
Bare mica	49.1	1997.4 ± 27.2	41	~0	NA	NA
PEI-coated mica	45.0	1323.4 ± 22.1	29	0.0177 ± 0.0003	1480 ± 77	33
STAI-coated mica[a]	47.8	747.1 ± 10	16	NA	NA	NA
	46.1	1603.6 ± 10	35	0.0248 ± 0.0004	1854 ± 523	40
MLV DOPC multilayer on bare mica[b]	48.3	1259.4 ± 41.9	26	0.0252 ± 0.0003	1581± 384	33

Detailed analysis of these Kiessig fringes requires consideration of the crystal truncation rods of mica[39, 43], and will be presented in a further publication[46].

For the DOPC multilayers prepared from a MLV dispersion, the Bragg peaks are absent in the XRR curve collected 20 min after water addition (Figure 5D, E), suggesting a less stable film as compared to the SUV sample. Similarly, Kiessig fringes indicate the presence of a bilayer at the mica-water interface of thickness d ~ 44.1 Å[46], in close agreement with that for the remnant bilayers in the SUV sample under water (~45.3 Å). Our results indicate that, in addition to a more ordered structure, the multilayers from the SUV dispersion showed better stability against water, probably due to fewer structural defects where delamination of the multilayers would be initiated. We have also subsequently incubated the sample with a DOPC SUV dispersion; however, the multilayers were not re-formed in the solution (*cf*. Figures S3D, E in ESI). In addition to the finite crystalline domain size as characterised by the coherence length L_a, the lattice spacing fluctuations (*i.e.* the paracrystalline disorder) may also contribute to the broadening of the Bragg peaks[55], which is consequently accompanied by a decrease in the peak intensity. This originates from the crystallites with slightly different *d*-spacing values, manifesting in increased broadening of the Bragg peaks with increasing diffraction orders n. This is evident from the reduced L_a values in Table 1 for higher order peaks (n = 3, 4) in the case SUV DOPC multilayers on mica. This means that the Scherrer equation is insufficient on its own to fully describe the structural order due to bilayer thickness fluctuations in the multilayer film, and a correction should be applied. The two contributions from the crystallite size and the distortion caused by the paracrystalline disorder may be separated by plotting the broadening of each peak ($(\Delta Q)^2/(2\pi)^2$) versus the fourth power of the diffraction order (h^4) for a plane of Miller index (*h*00).[47, 56, 57] From the linear fit to the plot, $y = y_0 + mh^4$, the coherence length, L_a, and the degree of disorder in the crystal, g, can be inferred respectively from the intercept y_0 and the slope m as

$$g = \frac{1}{\pi}\left(md^2\right)^{1/4}, \quad (3)$$

and

$$L_a = (y_0)^{-1/2}. \quad (4)$$

Figure 6 shows such a plot for the SUV DOPC multilayers in air on mica, PEI- and STAI-coated mica, as well as the MLV multilayer sample, for which at least three orders of reflections have been observed to allow this plot.[55] For SUV multilayers on mica, the bilayer thickness fluctuation is small and thus the slope of the plot is null (m ~ 0). The paracrystalline disorder parameter g (*cf*. Table 4) for the multilayer on the STAI-coated mica (0.0248 ± 0.0003) is greater than that for the PEI-coated sample (0.0177 ± 0.0007), and the MLV sample showed a degree of paracrystalline disorder (g = 0.0252 ± 0.0003) similar to that on STAI. The conclusion on the structural order from such an analysis thus concurs with that by the Scherrer equation analysis. The obtained L_a values, also listed in Table 4, are comparable to those obtained from the Scherrer equation. Overall, the multilayers prepared from the SUV dispersions are more ordered than those from the MLVs, with larger crystalline domain sizes and smaller bilayer thickness perturbations. The number of layers m calculated using values obtained from Equations (3) and (4) are reported in Table 4, which are slightly higher than those obtained from the Scherrer equation, but follow the same trend.

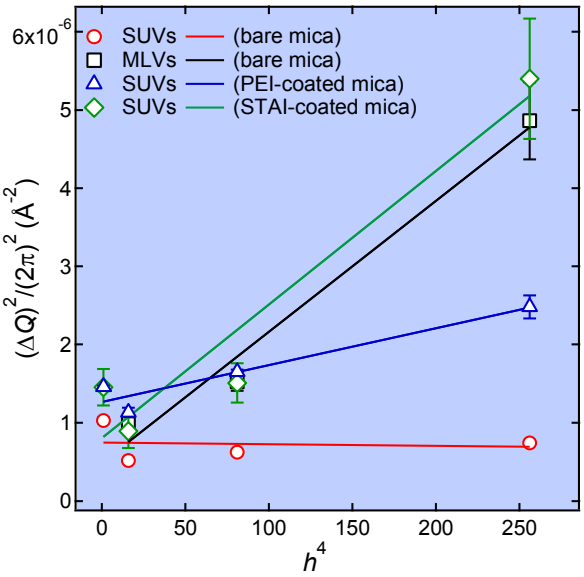

Figure 6: Peak broadening $(\Delta Q)^2/(2\pi)^2$ of lamellar reflections as a function of the fourth power of the diffraction order h^4 for DOPC multilayers from MLVs on bare mica (squares), and SUVs on bare mica (circles), on PEI-coated mica (triangles), and STAI-coated mica (diamonds). From the slopes m of the liner fits ($y = y_0 + mx$), the paracrystalline disorder parameters g could be calculated from Eq. (3). From the intercept y_0, the coherence length L_a could be estimated from Eq. (4). These values are listed in Table 4. The error bars for the SUV multilayers on mica are smaller than the circles and thus invisible.

4. Conclusions

In the present work, DOPC multilayers have been prepared by drop casting aqueous dispersions of liposomes on different substrates, and the multilayer structure has been studied using the synchrotron XRR technique, facilitated by a unique "bending mica" method[43]. In general, ordered lamellar structures consisting of DOPC bilayer domains have been obtained, with the lattice planes aligned approximately parallel to the substrate, evident from the distinct Bragg diffraction peaks in the XRR curve. The Bragg peaks have been analysed, using both the Scherrer equation and in terms of the degree of paracrystalline disorder, to yield detailed structural information. The multilayers prepared from monodispersed, well defined small unilamellar liposomes/vesicles (SUVs) ~ 90–100 nm in diameter were

more ordered than those from multilamellar liposomes/vesicles (MLVs). This manifested as a larger coherence length (or domain size) perpendicular to the substrate, and an almost zero paracrystalline disorder parameter, which indicates little fluctuations in the bilayer thickness throughout the thin film. The MLV multilayers showed polymorphism, with multiple Bragg peaks observed, exhibiting a number of different bilayer thicknesses.

The SUV multilayers also showed stronger stability against water as compared to the MLV multilayers, retaining the Bragg peaks in water for up to 2 hours. This observation again points to a more ordered structure with fewer defects in the domains, where delamination of the multilayer would be initiated. Relative fast XRR scans allowed us to study the rapid structural transition as the multilayers were submerged under water. We found that bilayers became swollen rapidly, with a hydration layer of thickness corresponding to 4-5 water molecular layers per bilayer.

We have also found that the surface chemistry of the substrate affected the multilayer structure. The multilayers cast on PEI- and STAI-coated mica from DOPC SUV dispersions had a higher degree of paracrystalline disorder than those on bare mica, with the STAI-coated mica, more hydrophobic compared to bare and PEI-coated mica, showing a greater effect, leading to structural polymorphism in the multilayers. This could be attributed to the interactions between the rupturing liposomes with the surface layer, dominating lipid packing immediately adjacent to the interface, which interestingly was templated by the bilayer domains subsequently formed, with the bilayer structure retained throughout the film. This highlights the importance of the substrate chemistry, and conversely offers a mechanism to tune the multilayer structure by controlling the surface charge and wettability.

Previously, lipid multilayers have been typically prepared from an organic solvent, due to the extremely low solubility of lipids in water. We suggest that DOPC liposomes serve a dual role. They act as a delivery matrix – here an appreciable lipid concentration in water (up to ~25 mg mL^{-1} or 14 mM) was feasible. In addition, they serve as a structural precursor, with their lamellar structure readily retained upon rupture at the solid surface upon evaporation to facilitate rapid multilayer formation. Pre-forming such liposomes in aqueous media would also offer a mechanism to incorporate desired functional ingredients in the lamellar structure, which could be readily transferred to the multilayers. Other potential variables such as controlling the evaporation rate *via* temperature and using solvent mixtures could offer further mechanisms for tailoring the multilayer structure and await future exploitation. Our results represent first detailed structural characterisation of lipid multilayers *via* the pathway of drop casting aqueous liposome dispersions, pointing to controlled preparation of ordered lipid multilayers by tailoring the liposome homogeneity and substrate surface properties, potentially offering a simple method for inclusion of hydrophilic and hydrophobic functional additive (*e.g.* drugs or nanoparticles; Sironi *et al.*, in preparation[46]) in lipid multilayer based hybrid materials.

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Structure of lipid multilayers *via* drop casting of aqueous liposome dispersions

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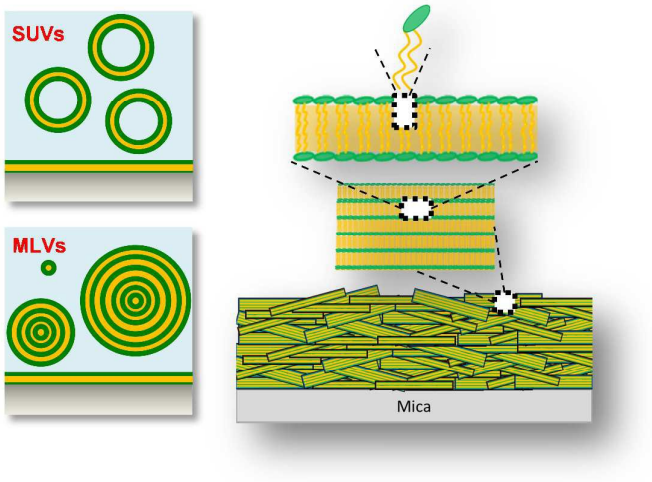
Liposomes serve as a lipid delivery matrix and a structural precursor, facilitating solid-supported lipid



multilayer formation via simple drop casting.