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Article

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Langmuir, **Just Accepted Manuscript** • DOI: 10.1021/acs.langmuir.7b02076 • Publication Date (Web): 17 Aug 2017

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Abstract

Water in oil (w/o) simple emulsions are dispersed micro-confined systems which find applications in many areas of advanced materials and biotechnology, such as food industry, drug delivery and cosmetics, to name but a few. In these systems, the structural and chemical

properties of the boundary layer at the w/o interface are of paramount importance to determine functionality and stability. Recently, microfluidic methods have emerged as a valuable tool to fabricate monodisperse emulsion droplets. Due to the intrinsic flexibility of microfluidics, different interfaces can be obtained, and general principles governing their stability are needed to guide the experimental approach. Herein, we investigate the structural characteristics of the region encompassing the liquid/liquid (L/L) interface of w/o emulsions generated by a microfluidic device in the presence of the phospholipid 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and other intercalating amphiphiles (dopants), using microfocused small angle X-rays scattering (μ-SAXS). We show that phospholipids provide a stable and versatile boundary film of ~100 μm, whose basic units are swollen and uncorrelated DMPC bilayers. The internal arrangement of this interfacial film can be tuned by adding molecules with a different packing parameter, such as cholesterol, which is able to increase the stiffness of the lipid membranes and trigger inter-bilayer correlation.

KEYWORDS Liquid/liquid interface, simple emulsions, micro-SAXS, microfluidics, DMPC membranes.

Introduction

Interfaces are the boundary layers which separate different bulk regions of matter, controlling their mutual confinement and boundary properties, such as interfacial tension and partition coefficients of solvated species. With respect to bulk phases, these layers present peculiar chemical, physical and biological properties that have drawn the interest of researchers from many different fields.[1] Simple emulsions, where aqueous droplets are dispersed in an oil phase, (w/o emulsions or, *vice versa,* o/w emulsions) are typical examples of systems exhibiting high interfacial area to volume ratio. These systems are well suited to study the crossing of chemical species between liquid regions with different hydrophilic character and curved interface. Moreover, simple emulsion are the object of great practical interest, since they have found applications in many technological areas, including food industry, pharmaceutics and cosmetics.[2] Generally, w/o and o/w emulsions are thermodynamically unstable, and surfactants, such as biocompatible phospholipids,[3,4] polymers[5] or colloidal particles,[6,7] can be used as stabilizing agents. In particular, phospholipids are an attractive class of compounds for biomedical applications and they have been used as emulsion stabilizers in drug delivery.[8,9,10,11] The majority of these works report on o/w droplets, which are well suited for enhancing bioavailability of poorly water soluble drugs. However, high resolution (nm or molecular level) studies are difficult to be performed, due to the complexity and limited stability of these systems. The kinetics of droplet evolution has been thoroughly studied, since it governs the stability of the system toward coalescence.[12,13]

As for the structural and chemical properties of droplet assemblies, it is well established that the amphiphilic layer at the liquid/liquid interface plays a key role in determining both the emulsion stability toward coalescence,

and the permeability of different species solvated in the bulk regions. The rigidity and lateral homogeneity of lipid bilayers can be modified by adding sterols or other molecules with a rigid core, which are able to induce the formation of rafts and defects in the lipid matrix. Sterols are also considered suitable components of artificially made bilayers, since they occur naturally in biological membranes.[14,15] In particular, cholesterol is a major constituent of the plasma membranes in eukaryotic micro-organisms, and its use allows to build up realistic models for cell membranes. Furthermore, it is interesting to investigate whether cholesterol perturbs the structure of the limiting layer in simple w/o emulsions, since the occurrence of defects in the interfacial membrane can influence the permeation of species able to undergo passive diffusion. The properties of the droplet surface depend not only on the nature and composition of the stabilizing layer, but also on the method used to build-up the interfacial film. Bulk emulsification procedures, involving drop breakup by shear or impact stress generated by mechanical agitation, have been described for the preparation of emulsions.[16,17] However, the obtained emulsions may present high dispersity, resulting in poorer performance and/or lower stability, thus limiting the extent of applications. Recently, microfluidics has emerged as a versatile technique to generate monodisperse emulsions with high throughput, and enhanced control over the emulsion design, in particular when the interface is constituted by a lipid-based layer.[18-20]

Here, with the aim to reach a deeper understanding of the structural properties of these systems, we investigated the structure of the region surrounding the liquid/liquid (L/L) interface of w/o emulsions generated by a microfluidic device in the presence of amphiphilic molecules, by using microfocused small angle X-rays scattering

(μ-SAXS). Previous studies combining microfluidic devices with X-ray focused beams have demonstrated, that the complementarity of the two techniques represents a powerful tool to follow up structural changes with high spatial and temporal resolution.[21,22,23] By using focused X-rays, raster scanning of the sample can be performed, allowing a comprehensive study of a heterogeneous sample with real-space resolution only limited by the beam size.[24,25,26] To the best of our knowledge this is the first study probing the L/L interface of a single droplet by μ-SAXS.

We show that phospholipids provide a stable and versatile boundary layer, whose properties can be tuned by adding dopants to modify the interface stiffness and charge.

The lipid layer was fabricated using dimyristoyl-phosphatidylcholine, with and without cholesterol added as a rigidity modulator. We also included a positive or a negative charged lipid dopant, since ionic lipids are known to affect the membrane lamellarity.[27-29]

Experimental section

Materials.

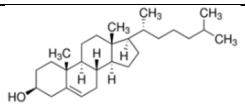
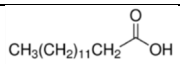
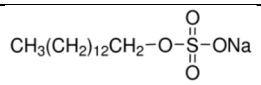
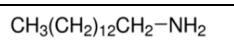
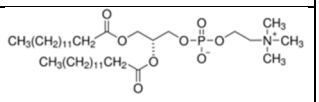
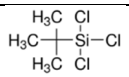
1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Lipoids Inc. Cholesterol (CHOL), myristic acid (Myr-A), sodium tetradecylsulfate (STS) tetradecylamine (TDA), *tert*-butyltrichlorosilane and polyvinyl alcohol (PVA) were all purchased from Sigma-Aldrich. The chemical structure of these compounds is reported in Table 1

Sample preparation

Simple water-in-oil (w/o) emulsion droplets were generated in a microfluidic device consisting of an inner polyimide coated 365/75 μm (outer diameter [OD]/inner diameter [ID]) borosilicate capillary tube, inserted in an outer 1/0.5 mm (OD/ID) borosilicate capillary tube.

A coaxial flow was obtained by pumping in the outer tube a mixture of an oil phase made by chloroform/cyclohexane 1:2, v/v and 1% w/w DMPC, flow rate of 20 μL min^{-1} (flow rate = 10 μL min^{-1}), while the inner tube, contained an aqueous 2% w/w PVA solution.

Table 1. Chemical structure the compounds used in this work

Commercial name	Chemical structure
Cholesterol	
Myristic acid	
Sodium tetradecylsulfate	
Tetradecylamine	
DMPC	
Polyvinyl alcohol	
tert-butyltrichlorosilane	

This resulted in separated w/o emulsions. PVA was added to the aqueous phase, since it is a well-known viscosity modulator frequently used in microfluidics, for example in generation of more complex emulsions such as w/o/w double emulsions. Hence, considering comparison with the broad variety of systems using this viscosity modulator, PVA was also applied here. The droplets were collected in borosilicate capillaries of 0.3 mm (ID) and 10 μm wall thickness, in order to minimize the parasitic signal from the glass background. To obtain concave w/o interfaces, the inner walls of these tubes were rendered hydrophobic by filling them with a 2% (w/w) solution of *tert*-butyltrichlorosilane in toluene. After 2h, the tubes were rinsed with toluene, and oven dried at 70 ºC for other 2h.

Microfocus SAXS data acquisition and analysis

Scanning micro-X-ray diffraction was performed at the ID13 beamline of ESRF (Grenoble, France). A monochromatic X-ray beam (λ = 0.095372 nm) was focused down to a 1.5 × 1.5 μm^2 spot at the desired sample position, using a combination of Beryllium-

compound refracting lenses (Be-CRL transfocator). The sample to detector distance was 0.941 m, corresponding to a q-range of $0.2 < q < 8$ nm^{-1}, with q being the scattering-vector defined as $q = (4\pi/\lambda) \sin(\theta/2)$ and θ is the scattering angle, i.e. the angle between the scattered and incident radiation. One- or two-dimensional beam raster scans covering the emulsion interfaces were performed in transmission geometry. At each raster step, a scattering pattern was collected by the DECTRIS EIGER 4M single photon counting detector, providing frames of 2070 × 2167 pixels (75 × 75 μm^2 pixel size) at rates up to 750 Hz. Radiation damage was observed after about 0.2 s irradiation. Exposure times were hence limited to 0.1 s/raster point. Microfocus SAXS was used to investigate the the lipid layer surrounding simple emulsion droplets. 1-dimensional raster scans, with a step size of 2 μm, were performed in a direction axial to the capillary tube, covering a region from 0.7 mm to 0.7 mm, with the droplet center as zero-reference point. This allowed investigating the microemulsion across both w/o interfaces. An on-line microscope with focal spot coinciding with the X-ray was used to visualize the droplets, and design the raster scans. The 2D-diffraction patterns were azimuthally integrated to obtain 1D scattering intensity profiles ($I(q)$ vs q), by using the SAXS utilities package.[30]

Results and discussion

The boundary lipid layer of simple w/o emulsions was probed by scanning across the interface with μ-SAXS. **Figure 1** shows the image of a typical droplet confined in the capillary (top). The corresponding imaging diffraction of the droplet interface is also represented (bottom). The scattering contribution from the capillary was subtracted in order to highlight the scattering signal from the water/oil interface. At a first glimpse, the 2D scans did not evidence any preferred orientation; this allowed excluding alignment

of the sample due to shearing effects once loaded in the capillary. The integrated intensity profiles recorded in the oil phase (containing pure DMPC or DMPC/CHOL), and in the water/PVA phase did not show any excess scattering, as typically observed for correlated or uncorrelated structures **(Figure 2)**, indicating that both the oil and water regions were isotropic media with no dispersed aggregates. In principle, reverse micelles could be expected in the oil phase, due to partial lipid solubility in the chloroform/cyclohexane mixture, while the aqueous phase could contain mixed micelles of lipids and cholesterol.

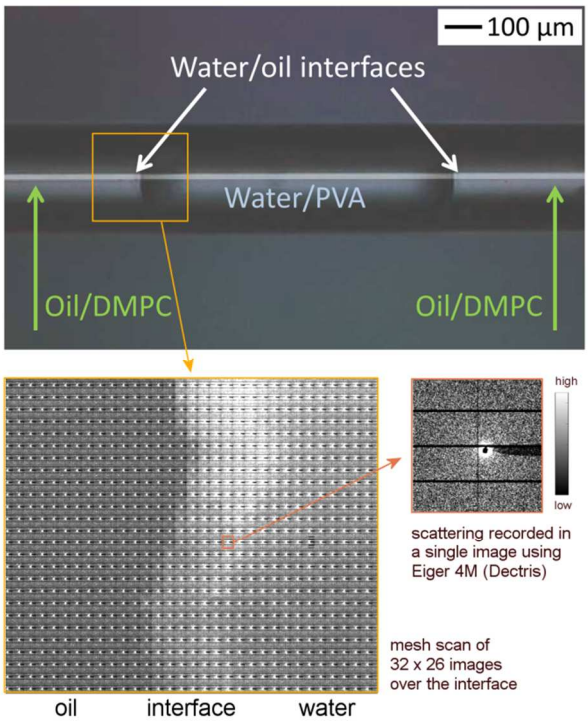


Figure 1. (top) w/o emulsion droplet of an aqueous solution of PVA (2%, w/w) in a mixture of chloroform/cyclohexane (1:2, v/v) containing 1% (w/w) DMPC, in a borosilicate capillary of 300 µm inner diameter, and wall thickness of 10 µm. (bottom) 2D reconstruction of the droplet interface by imaging diffraction.

In sharp contrast to the inner and outer phases, the boundary layer was characterized by a diffuse scattering pattern, which extended for ~100 µm. The SAXS intensity profile along this distance had a q^{-2} decay in the intermediate q-range, a typical behavior of locally flat scattering objects. In this case, the scattering objects were localized at the interface, and were isotropically arranged over a mesoscopic scale of about 100 µm. In addition, the absence of a correlation peak in the SAXS patterns of the water/DMPC/oil system, was a clear indication that no (or very weak) correlation among these bilayers occurred in the w/o emulsions stabilized by pure DMPC. We could thus rule out the

possibility that in such a system, the basic structure was not a lamellar stacking (**Figure 2**, black filled triangles). In fact, if oligolamellar organization was present even in fractions as low as 3-5 % of the total lipid concentration, the extension of the raster beam would allow observing quasi-Bragg peaks superposed to the diffuse scattering of uncorrelated bilayers. The addition of moderate cholesterol (20% mol/mol) content to the oil/DPMC phase, resulted in the appearance of a fairly broad peak at ~1 nm^{-1} in the SAXS intensity profile (**Figure 2**, blue empty circles), which corresponded to a distance of 0.60–0.65 nm in the direct space. This is the characteristic repeat distance of DMPC lamellae,[29,31] and indicated that cholesterol is able to induce marked stiffening of the bilayers, thus promoting inter-bilayer correlation within the interfacial membrane. Here, the contrast of the X-rays is at maximum, and the strong intensity indicated lipid self-assembly. For comparison, the scattering profiles of different references are also shown in **Figure 2**.

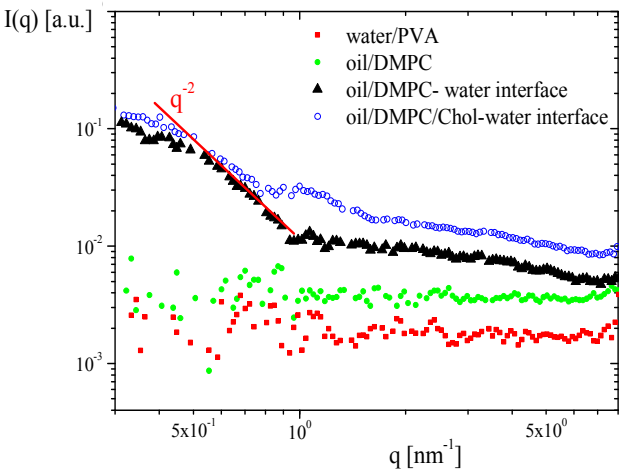


Figure 2. SAXS intensity profiles for simple w/o emulsion systems. The water/2% w/w PVA (red filled squares) and the oil/1% w/w DMPC (green filled squares) profiles represent bulk solutions far from the interface. The oil/1% w/w DMPC profile (black filled triangles) was recorded ~50 µm from the w/o interface. The q^{-2} power decay is shown in the Figure. The blue empty circles represents the same scenario with 0.8/0.2 mol/mol DMPC/cholesterol (DMPC/CHOL).

The intensity recorded for each frame at the q-value of 0.5 nm^{-1} is reported in **Figure 3,** as a function of the scanned distance. The resulting profile clearly shows, that the increase in scattering intensity at this chosen q-value was extended for ~100 µm into the

continuous (oil) phase. After the subtraction of the water background signal, the SAXS intensity patterns were fitted with the SasView freeware package.[32] The best-fit profiles for the oil/DMPC-water (black filled triangles) and oil/DMPC/CHOL-water (blue empty circles) systems are shown in **Figure 4**, and correspond to the following best-fit parameters:

Scattering length density (SLD x 10^{-6} Å^{-2})

SLD heads = 10.6 ± 0.5

SLD tails = 4.0 ± 0.5

Head thickness = 4 ± 0.2 Å

Tail thickness = 40 ± 1 Å

Tail polydispersity = lognormal distribution with standard deviation σ = 0.12

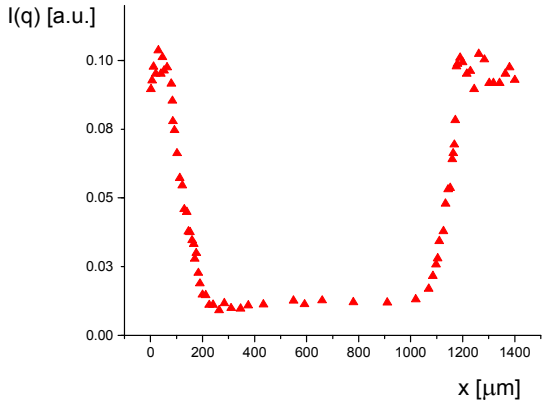


Figure 3. SAXS intensity at q = 0.5 nm^{-1}, recorded at an axial line covering both the w/o interfaces of the simple w/o emulsion system, showing an increased scattering intensity upon crossing the interfaces. The zero-reference point refers to the center of the aqueous drop.

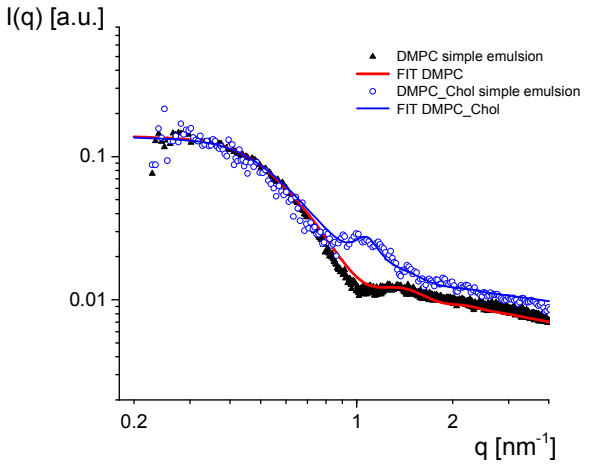


Figure 4. SAXS intensity profiles for the simple w/o emulsion system. The (black filled triangles) oil/1% w/w DMPC profile was recorded ~50 µm from the w/o interface. The blue circles represent the same scenario for the system 4/1 mol/mol DMPC/cholesterol (DMPC/CHOL, blue empty circles). The best fit (solid lines) in each case was obtained with the parameters reported in the text. Water signal was subtracted from the scattering curves.

The best fit value obtained for the total bilayer thickness (~ 88 Å) indicated extensive swelling of DMPC, since this value is almost twice as high compared to values reported in literature describing liposomes or lamellar

phases in aqueous media.[33-36] This finding, together with the low scattering length density (SLD) of the tails, was consistent with DMPC monolamellar membranes that included cyclohexane in their inner hydrophobic core. In the presence of cholesterol at 20% mol/mol, the scattering profile was satisfactorily reproduced by introducing a model independent correlation peak with a Lorenzian shape (HWHM = 0.13) centered at 1.07 nm. As mentioned above, this peak indicated that cholesterol induced partial ordering in the bilayer arrangement. We chose to perform this phenomenological description rather than a more detailed analysis, due to the numerous adjustable parameters in this multicomponent system, which might result in data over-interpretation. Indeed, to give quantitative information on the changes brought about by cholesterol in the structural parameters, a range of different molar ratios should be taken into account. The observed effect, however, is well in agreement with the rigidification usually exerted by sterols in fluid lipid membranes, such as DMPC bilayers at our working conditions.[31-33]

The scattering intensity profiles of DMPC bilayers containing dopant amphiphiles, *i.e.,* the curvature/surface charge modifiers Myr-A, STS, TDA at 20% mol/mol content, are shown in **Figure 5**. The structural features observed do not significantly differ from the case of pure DMPC, suggesting that the main bilayer properties are retained after inserting these intercalating agents in the lipid layers. Noteworthy, still no sign of aggregation was detected either in the external (oil) or internal (water) micro phase of the droplets. The overall profiles of the systems doped with ionic surfactants were thus similar to those reported in **Figure 3**. This proved that changing the bilayer composition by replacing 20% mol of DMPC with MyrA, STS or TDA did not markedly affect the lipid

arrangement at the interface, nor the bilayer swelling.

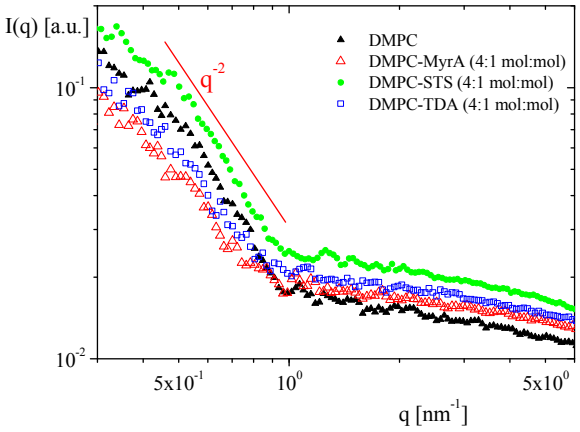


Figure 5. Scattering intensity profiles of pure DMPC and composite bilayers present at the oil/water interface. The ratios DMPC/Myr-A, DMPC/STS and DMPC/TDA are all 0.8/0.2 mol/mol.

Summary and Conclusions

Simple w/o emulsions represent a useful model for the studies of the structural properties of phospholipid membranes at the water/oil interface, and can be used to optimize emulsion systems applied in food industry, pharmaceutics and cosmetics, and get insight on chemical communication among liposomes.[37,38] Furthermore, they provide information on the issue of emulsion stabilization with amphiphilic molecules and on the possibility to tune the properties of the interface. This is a relevant subject, due to the wide range of emulsion applications. Here, we studied the structural organization of DMPC-based layers surrounding aqueous droplets in an oil phase by Microfocus-SAXS measurements. In particular, we built monodisperse droplets by using a microfluidic device, and we modulated the stiffness and charge of the boundary lipid layers by adding amphiphilic dopants. The analysis of SAXS intensity profiles showed that at the oil/water interface lipids were self-assembled, and arranged into bilayer-based structures. On the contrary, no (inverse or direct) micelles were found in the bulk oil and water phases. Overall, the lipid layer had a thickness of ~ 88 Å, and was constituted by swollen DMPC. This extended layer almost lacked internal organization among bilayers. Therefore, we can speculate that phospholipids are arranged at the surface of a

simple emulsion droplet either as L_3 sponge phase[39], or as polydisperse unilamellar vesicles[40]. We also found that the addition of cholesterol increased the bilayer rigidity, resulting in partial ordering and inter-bilayer correlation, as it can be expected for sterols intercalating a Lα lipid phase. On the contrary, the presence of amphiphilic dopants, such as Myr-A, STS and TDA, did not induce marked modifications to the DMPC layer covering simple emulsions droplets. These results will be useful for future emulsion engineering by single droplet manipulation. Moreover, the presence of residual organic solvent in the phospholipid bilayers, which is the cause of marked swelling, is sure to have an effect on the permeability of the interface, since it can facilitate the crossing of more hydrophobic species. The combined use of micro-focused beam scattering techniques with microfluidics, is undoubtedly of interest for the community of researchers working with w/o emulsions stabilized by lipids. We thus believe, that this work can help advancing the field of emulsion formulation, and contribute to comprehend specific aspects of communicative systems where lipid membranes surround water confined microdroplets.[41,42]

ACKNOWLEDGMENT The authors thank the staff of ID13 (ESRF Grenoble) for their guidance and keen interest throughout this work

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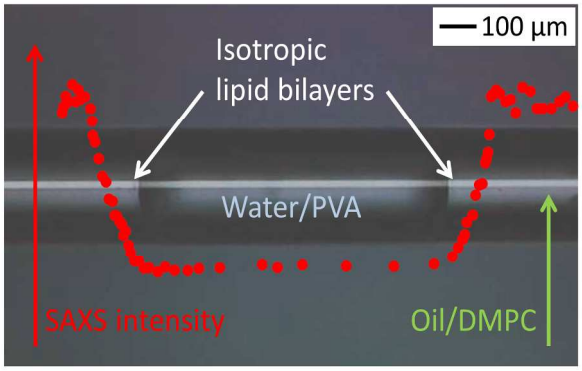

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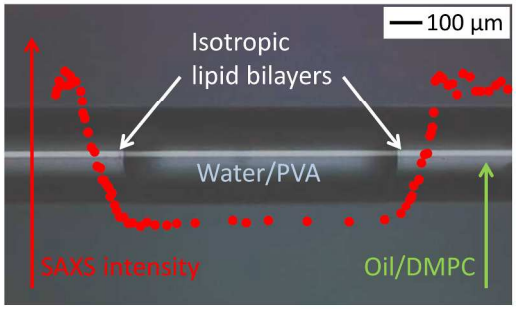

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