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BRIEF RESEARCH REPORT
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Structural Evidence for a Reinforcing Response and Retention of Hydration During Confinement of Cartilage Lipids

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Lipids have an important role in the complex lubrication of articulating joints, however changes in lipid phase behavior that occur owing to mechanical confinement are not well understood. Here, a surface force-type apparatus has been combined with neutron reflectometry to measure confinement-induced changes in the structure of lipids, the major surface-active component of the lubricant in articulating joints. The same incompressible state was accessed under low uniaxial stress (1 bar), irrespective of whether the lipids had started out unconfined above or below the L_α phase transition, and irrespective of whether they were fully or partially hydrated. In this incompressible state, the lipid component had thickened indicating extension and rearrangement of the lipid chains in response to the applied stress. The small amount of water remaining between each lipid bilayer was found to be similar for all chain lengths and starting phases. This represents the first structural evidence of the tightly bound water layer at the headgroups, which is required for hydration lubrication under load.

Keywords: phosphatidylcholine, neutron reflectometry, compression, dehydration, phase change, lubrication

INTRODUCTION

In contrast to mechanical devices, which are mostly lubricated with oil, nature lubricates exclusively with water. In fact, water-based lubrication presents several advantages as water is nontoxic, abundant and an effective coolant, but water alone is a poor lubricant. It has been extensively shown that nature overcomes this with the addition of biological molecules, with the ability to modify surfaces to make them far more lubricating at slow speeds and under the high loads experienced in human joints [1, 2]. However, the mechanisms underlying biological aqueous lubrication in confined conditions are far from being well understood.

There have been a number of studies on the role of both lubricin and hyaluronic acid (HA) within the synovial fluid for lubrication [3, 4] and antifouling [5]. With increasing complexity, phospholipids have been included with the lubricin and HA in surface force measurements [6, 7]. A lowering of the friction coefficient was found when all components were included, with the phospholipids interpreted to be located on the outer surface of the lubricin-HA layer. It is still unclear exactly in what form the phospholipids within the synovial fluid exist, they could form vesicles [6, 8], alternatively some authors propose that a single surfactant like layer forms on each cartilage surface

[6, 9] or they could form bilayers or multilayers on the surfaces. The Hills model focusses on the role of the phospholipids at the articular surface of cartilage, it describes phospholipids to be the major "solid" component of the lubricant in articulating joints [10–12], where the fluid component is water. The breakdown of this complex lubricating layer can contribute to wear of the cartilage and eventually osteoarthritis [13]. Thus, the structural study of these lipid constituents under confinement can offer insight into their role in lubrication.

Investigations into the force response of individual lipids under confinement is well established using the surface force apparatus (SFA) [14–17]. In particular, Orozco-Alcaraz and Kuhl [14] applied the technique to lipids while using silica as the substrate, to study symmetric DPPC-DPPC interactions and understand the influence of substrate charge on the bilayer interactions in confinement. Although the SFA provides a very sensitive measurement of forces in friction and confinement [18, 19], and can be used to infer the interactions between bilayers [15, 20], it is not possible to measure the structure simultaneously while the confinement is applied. Another confinement approach is pipette aspiration, which showed that the area of saturated lipids expands with applied confinement [21]. While the increase in area is measured directly, the full structure cannot be elucidated using the technique. Other pressure related approaches to confinement have also been used including osmotic pressure probed using small angle X-ray scattering [22, 23], and also through molecular dynamics simulations [24, 25]. X-ray and neutron reflectometry have proven especially powerful tools in the study of biomembranes in order to measure structure. For example, they can be used to determine phase behavior, layer spacing and volumes occupied by headgroups [26, 27]. These scattering approaches are particularly useful for the study of confinement [28]. Here, we utilize a new type of confinement apparatus [29–32] combined with *in situ* neutron reflectivity. It is important to mention that unlike a traditional SFA the samples are equilibrated at a small number of set pressures, with the distance between the confining surfaces, and the structure and hydration of the lipids, then determined by neutron reflectivity. In our experiments we study zwitterionic lipid stacks as a model for articulating cartilage surfaces under uniaxial confinement. Thus, direct measurement of the structure during confinement is undertaken.

METHODS

Materials: The lipids used were a series of saturated phosphatidyl choline lipids with increasing hydrocarbon chain length; 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC, 12:0, T_m = −2°C), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, 14:0, T_m = 24°C), 1,2-palmitoyl-snglycero-3-phosphocholine (DPPC, 16:0, T_m = 41°C), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, 18:0, T_m = 55°C) and one with an unsaturated chain 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC, 18:1c9, T_m = −17°C). All lipids were purchased from Avanti Polar Lipids Inc. (United States) distributed by INstruchemie BV (Netherlands) and the transition temperatures from the LIPIDAT NIST standard reference database [33]. Chloroform (>99.9%, HPLC grade) and deuterium oxide were supplied by Sigma Aldrich (United Kingdom and France). Demineralised (Milli-Q) water with a resistivity of 18.2 MΩ cm was used in cleaning the silicon surfaces.

Sample preparation and experiment conditions: Samples were prepared by spin coating onto 75 mm diameter silicon blocks from a solution of 0.5% lipid in chloroform at 3000 RPM and annealed at 50°C in an oven for 1 h before use. The silicon blocks were secured in the confinement apparatus, **Supplementary Figures S1** in Supporting Information (full details [29]) and mounted vertically on the D17 reflectometer [34]. D_2O was used to hydrate the hydrogenous lipids. Two different hydration methods were used: vapor hydration was achieved by placing ~2 ml of D_2O inside the confinement cell but not where it would directly touch the sample; full hydration involved placing a few drops of D_2O onto the surface of the sample before inflating the flexible membrane against it to create the confined geometry [35–37]. Owing to the vertical sample geometry of the D17 reflectometer, fully hydrated samples could not be measured without confinement applied, instead measurements commenced at 1 bar of confinement. The vapor hydrated samples reflectivity measurements were taken without mechanical confinement (0 bar of confinement) before the membrane was inflated and then measurements were taken with 1, 3 and 5 bar applied [35–38]. With each confining stress increase, reflectometry measurements were repeated until the sample had equilibrated. A wide range in scattering vector *Q* was achieved using three different reflection angles between 0.4 and 2.8°. The size of the beam footprint on the sample was kept constant at 2.5 cm^2 × 2.5 cm^2 by adjusting the pre-sample slit sizes. Further details of the confinement cell, Melinex properties and control measurements indicating the efficiency of the apparatus for expelling D_2O can be found reproduced in the Supporting Information (**Supplementary Figures S2**) and in our previous publication [29].

Model fitting: A layer model was used to fit the neutron reflectometry data with the main repeat unit of a water layer and a lipid layer. A parameter, N_k, was included to estimate the variation in the number of bilayers in the lipid stack was used. The full details of the model, how the final model was developed and the parameters used are provided in the supporting information.

RESULTS

It has been shown that zwitterionic phosphatidylcholine (PC) lipids (>60% of all lipids) are a significant surface active (~13%) constituent of synovial fluid [5, 23], forming a stack of approximately 3–7 bilayers at the cartilage surface [39]. We model these surface lipids using stacks of lipid bilayers with PC head groups and a range of saturated chain lengths from dilauryl (12:0) to distearyl (18:0) chains; the lipids are spin coated onto silicon substrates. These layers were hydrated within the confinement sample environment using D_2O vapor and the

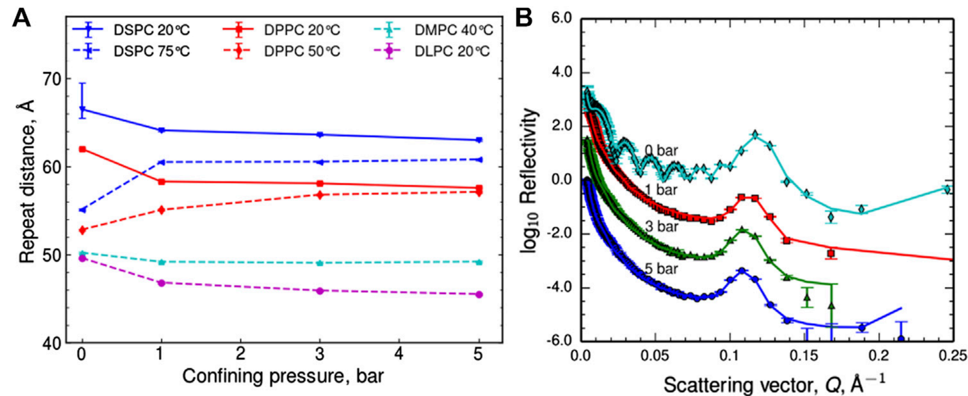

FIGURE 1 | (A) Trends of the repeat distances in the lipid bilayer stacks, hydrated with D_2O vapor taken from the positions of the Bragg peaks. Lines are to guide the eye only, with solid lines for temperatures below the expected phase transition to L_α and dashed lines for those above. **(B)** Neutron reflectometry profiles for DPPC hydrated with D_2O vapor at 50°C under different applied uniaxial stresses, off-set for clarity, the lines represent model fits to the data.

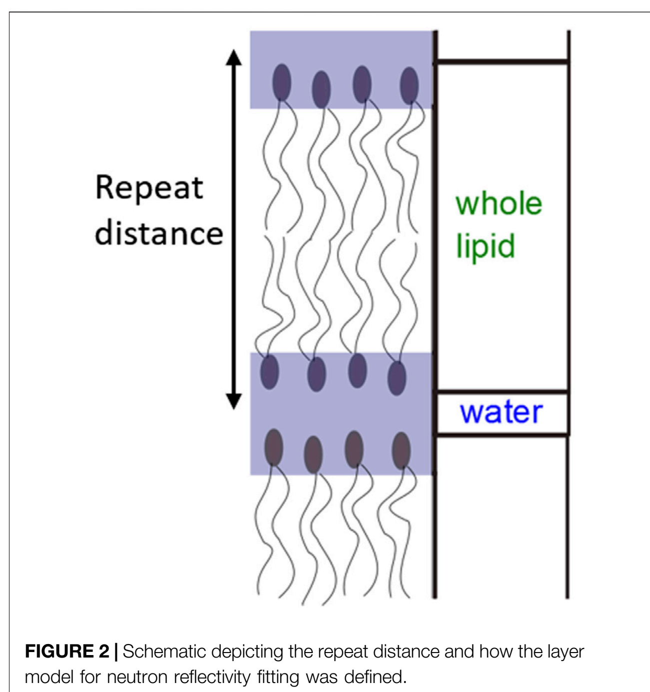

FIGURE 2 | Schematic depicting the repeat distance and how the layer model for neutron reflectivity fitting was defined.

neutron reflection measurements made using the D17 reflectometer (ILL, Grenoble) [34] uniaxial stress increasing from 1 to 5 bar (see [29], **Supplementary Figures S1**, Supporting Information and below for further experimental details).

Figure 1A shows the change in repeat spacing of the lipid bilayer stack (defined on the schematic in **Figure 2**), calculated from the Q position of the Bragg peak as an initial indication of the trends, as each sample was confined (data shown in Supporting Information, **Supplementary Figures S4–S5**). The 0 bar repeat distances were measured *in situ* prior to confinement at ~98% humidity. The values are similar but predictably slightly lower than literature values for fully hydrated stacks [40–42]. In most cases, the lipid stacks follow the trend of reducing their repeat spacing in response to confinement, as expected due to a loss of water from between the bilayers. However, for the DPPC and DSPC samples heated to above their phase transition and hence in the L_α phase, the opposite trend was observed and their repeat spacing in fact increased significantly on confinement. Further, in both cases repeat distances converge towards the same values on confinement as they each had for the lower temperature despite the different starting phases.

To extract further information from the neutron reflectivity curves, a layer model was used to fit the data. In the model chosen the lipid head and tail regions were combined into a layer with the water in between the lipids considered as a second layer so the repeat unit was one lipid bilayer and one water layer (**Figure 2**). Each lipid bilayer was defined by the same three parameters; thickness, neutron scattering length density (SLD) and roughness and the same three parameters were defined for the water layer between the lipid head groups. The outermost roughness and the variation in the exact number of bilayers across the surface of the sample were also taken into account during fitting (see also the schematic in Supporting Information, **Supplementary Figures S3**). **Figure 1B** shows this model fit to the DPPC at 50°C data set, full details of the model and parameters used to fit the data are given in the Supporting Information along with the complete data sets with corresponding fits (**Supplementary Tables S1–S5**, **Supplementary Figures S4–S7**).

The fitted thickness of these two layers as a function of confinement pressure can be seen for all of the samples in **Figures 3A,B**. This separation of the contributions to the overall repeat distance confirms that the water layer spacing is consistently reduced to 6 ± 2 Å after application of 1 bar confining pressure, independent of the lipid chain length or phase. This is consistent with a single solvation shell of water molecules closely associated with each PC head group [43], where 2.7 Å is the approximate diameter of a single water molecule [44] and also to the approach of protein solution scattering where proteins are modeled with a 3 Å bound water layer [45]. To

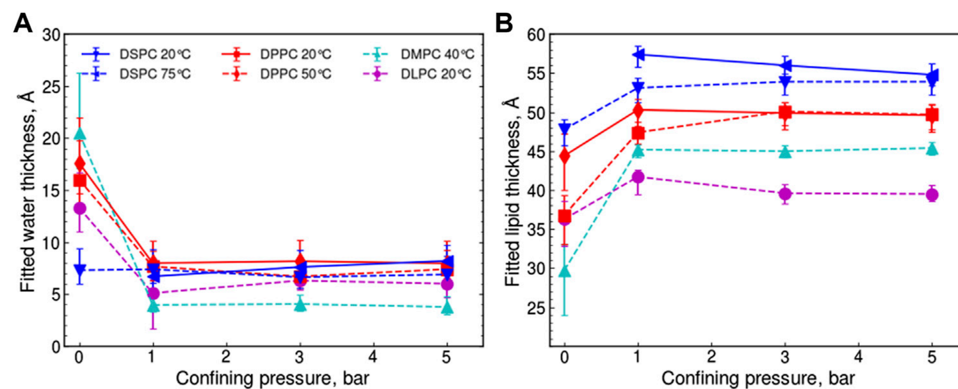

FIGURE 3 | Trends of the fitted **(A)** water layer thickness and **(B)** lipid layer thickness in the lipid bilayer stacks, hydrated with D_2O vapor, taken from the best model fits. Lines are to guide the eye only and the symbols define data for the same samples in both **(A)** and **(B)**. Uncertainties are derived from fitting the data with two alternative scattering length densities for the D_2O vapor which would not change within a series of confining pressures, meaning that the confidence in the relative positions of the points is greater than the error bars shown, this is discussed further in the Supporting Information.

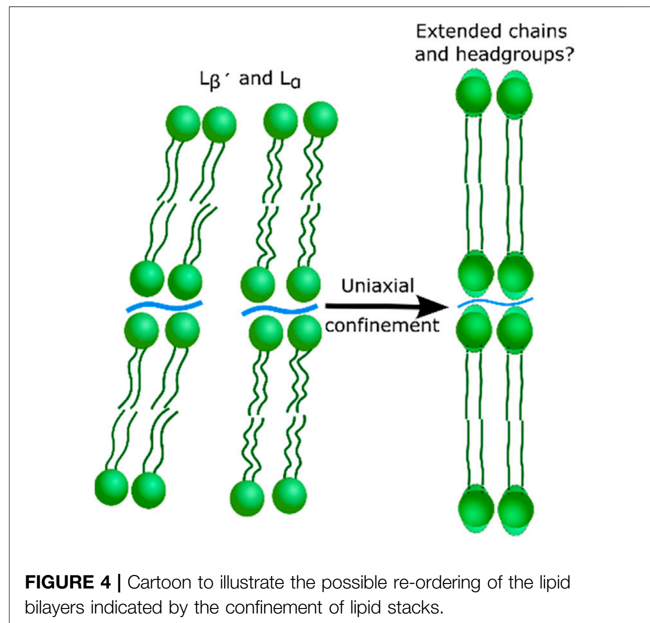

FIGURE 4 | Cartoon to illustrate the possible re-ordering of the lipid bilayers indicated by the confinement of lipid stacks.

ensure this phenomenon was not a function of hydrating the membranes using a humid atmosphere rather than bulk water, measurements on some lipid samples were repeated but hydrating with bulk water rather than water vapor (Supporting Information, **Supplementary Figures S6**). The water layer thickness also decreased to 6 ± 2 Å under 1 bar of confining pressure with no further loss of water at higher pressures, confirming that this was not due to limitations in the amount of available hydrating solvent.

In response to confinement by 1 bar, the thickness of the lipid bilayers (**Figure 3B**) increased for each lipid, independent of the tail length or starting phase, compensating (more or less) for the shrinking water layer. The same behavior was also seen for the fully hydrated samples (Supporting Information, **Supplementary Figures S6**). Further, the lipid bilayer thicknesses from the different starting phases converge at 3 bar for DPPC and 5 bar for DSPC suggesting that the same, incompressible, lipid state is accessed under confinement, irrespective of the starting phase. Thus, a direct correlation is observed between the amount of stress required to reach the incompressible confined state and the chain length of the lipid tails. It is possible that the response to confinement arises from the dehydration of the region between lipid bilayers, reducing the cross-sectional area per lipid headgroup and allowing closer packing of the lipid tail region resulting in a thickening, consistent with similar results seen for DLPC in X-ray diffraction studies [40].

Finally, while saturated PC lipids are the most prevalent constituent on the cartilage surface, a significant amount are also unsaturated, of which DOPC is present in the highest quantities [38, 46]. Therefore, for completion, these confinement experiments were repeated with stacks of DOPC bilayers hydrated with both vapor and bulk water. The analyzed data (Supporting Information, **Supplementary Figures S5, S6**) show the same features as in the saturated cases, despite the larger equilibrium area per molecule and relative disorder from the unsaturated double bonds on the lipids. The water spacing between bilayers reduces to ~6 Å, but the water was not expelled entirely, and the thickness of the lipid region swells correspondingly under confinement, both reaching a plateau by 3 bar of applied uniaxial stress. The ratio between the fitted water and lipid layer thicknesses (Supporting Information, **Supplementary Figures S8**) also shows a similar trend for all of the vapor hydrated lipids except for the DMPC, which starts from a more hydrated state, and the DSPC at 75°C, which starts from a less hydrated state which may be attributable to its denser tail region. However it is interesting that the saturation of the lipid tail does not cause a dramatically different behavior.

DISCUSSION

Here, we described the first direct structural study of lipid bilayers under confinement, providing an excellent model system for understanding the lipid lubricating properties of articulating cartilage. We propose that the low friction experienced between layers of stacked lipids as they slide past each other is due to the hydration lubrication mechanism, where a layer of water remains closely associated with a zwitterionic lipid head groups even at high pressures [47–51], in accordance with the Hills model [11, 12]. It should be noted that in this study the applied pressures were limited by our experimental setup to a maximum of 5 bar. Although a wide range of pressures occur at different points within a joint under various conditions starting from low pressures in the fluid [52, 53], like those probed here, and increasing to very high contact pressures (up to 200 bar) that can occur at contact points, locally within mammalian joints [54, 55]. In future experiments we hope to increase pressures to a range in line with the higher pressures that occur *in vivo* and particularly in diseased joints.

The results presented here show the first structural evidence to support our hypothesis, where a spacing approximately equivalent to one hydration layer of water molecules per lipid head group is maintained even at higher applied uniaxial stresses. SFA studies indicate that the lubrication behavior and frictional forces are maintained across a wide range of pressures [47, 48], and therefore the structures we measured at low levels of confinement remain comparable to those experienced locally at the cartilage surface [55].

Further, we have shown that the same lipid state corresponding to the lipid layer thickening is accessed under confinement, independent of the lipid tail saturation or starting lipid state. The thickening warrants further investigation but suggests a repacking of the lipid tail region due to the reduced cross-sectional area per lipid when the headgroups are partially dehydrated (as proposed in the illustration in **Figure 4**). The structure may be similar or identical to the sub gel phase [56, 57]. The results also offer an insight into the high mechanical robustness of the lipid bilayers. The results suggest that it is the access to a closer packed phase, with thicker layers for all lipids when confined, that increases the bilayer robustness. This lipid response is expected to improve their resistance to the high shear and compression forces experienced in human joints while still maintaining their hydrated lubricating properties. The water expelled during confinement may also play a role in the lubrication, synergistically with the transfer of fluid between the cartilage and synovial fluid which is believed to occur during joint lubrication [58]. Understanding of the hydration and structural changes of the PC lipids under uniaxial stress is the first step toward fully understanding how their structure is changed in dynamic, lubricating joints and how the act to improve the lubricating properties of synovial fluid. The composition of the synovial fluid changes with diseases such as osteoarthritis and DPPC has also been shown to reduce the friction coefficient and therefore is a prospective treatment for damaged joints [59, 60].

To conclude, we present the first measurements of the structures of lipid bilayers with a simultaneously applied uniaxial stress. The structural properties have been observed directly using neutron reflectometry, thus demonstrating that the apparatus used can provide extensive insight into the structural behavior of such biological systems. Specifically, in this study we have provided direct support for the mechanism of hydration lubrication previously hypothesized on the basis of indirect measurements.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material** and as raw data from the ILL DOIs referenced 35–37, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

LM: Conceptualization, Data curation, Investigation, Methodology, Original draft. SA: Investigation, Review and; editing. RB: Conceptualization, Investigation, Methodology, Review and; editing. WV: Conceptualization, Data curation, Investigation, Methodology, Review and; editing. SP: Conceptualization, Investigation, Methodology, Review and; editing, Supervision, Funding acquisition. RR: Conceptualization, Investigation, Project administration, Methodology, Supervision, Review and; editing, Funding acquisition.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphy.2021.703472/full#supplementary-material

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