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Interfaces: Adsorption, Reactions, Films, Forces, Measurement Techniques, Charge Transfer, Electrochemistry, Electrocatalysis, Energy Production and Storage

## Interactions between mutant bacterial lipopolysaccharide (LPS-Ra) surface layers: Surface vesicles, membrane fusion, and effect of Ca and temperature

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3 **Interactions between mutant bacterial lipopolysaccharide (LPS-Ra)**  
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6 **surface layers: *Surface vesicles, membrane fusion, and effect of  $\text{Ca}^{2+}$***   
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9 ***and temperature***  
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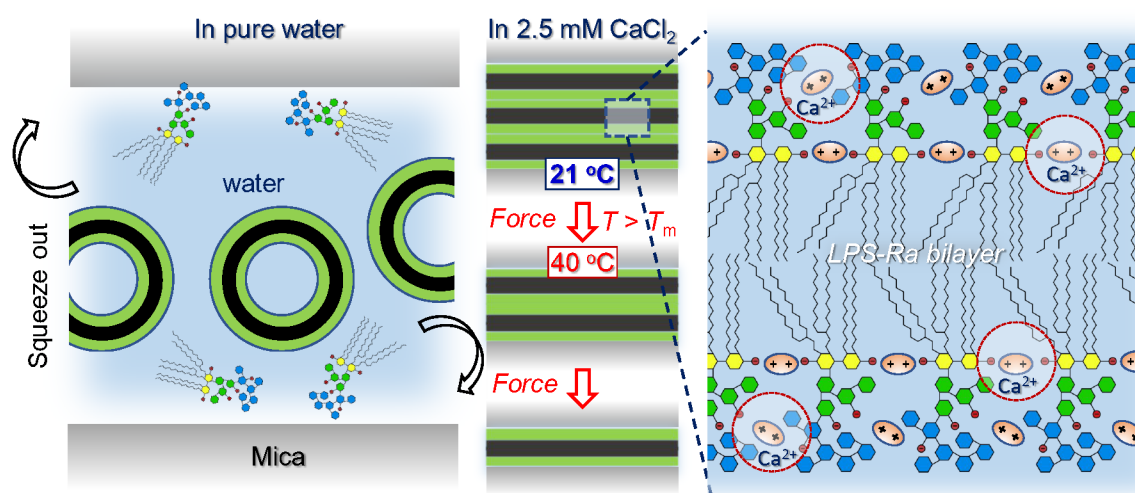
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56 **Abstract**

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3 Lipopolysaccharides (LPS) are a major component of the protective outer membrane  
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5 of Gram-negative bacteria. Understanding how the solution conditions may affect LPS-  
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7 containing membranes is important to optimising the design of antibacterial agents  
8  
9 (ABAs) which exploit electrostatic and hydrophobic interactions to disrupt the bacteria  
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11 membrane. Here, interactions between surface layers of LPS (Ra mutants) in aqueous  
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13 media have been studied using a surface force apparatus (SFA), exploring the effects  
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15 of temperature and divalent  $\text{Ca}^{2+}$  cations. Complementary dynamic light scattering  
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17 (DLS) characterisation suggests that vesicle-like aggregates of diameter  $\sim 28\text{-}80$  nm  
18  
19 are formed by LPS-Ra in aqueous media. SFA results show that LPS-Ra vesicle  
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21 adsorb weakly onto mica in pure water at room temperature (RT), and the surface  
22  
23 layers are readily squeezed out as the two surfaces approach each other. However,  
24  
25 upon addition of calcium ( $\text{Ca}^{2+}$ ) cations at near physiological concentration (2.5 mM)  
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27 at RT, LPS multilayers or deformed LPS liposomes on mica are observed, presumably  
28  
29 due to bridging between LPS phosphate groups and between LPS phosphates and  
30  
31 negatively charged mica mediated by  $\text{Ca}^{2+}$ , with a hard wall repulsion at surface  
32  
33 separation  $D_0 \sim 30\text{-}40$  nm. At  $40^\circ\text{C}$  which is above the LPS-Ra  $\beta\text{-}\alpha$  acyl chain melting  
34  
35 temperature ( $T_m = 36^\circ\text{C}$ ), fusion events between the surface layers under  
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37 compression could be observed, evident from  $\delta D \sim 8\text{-}10$  nm steps in the force-distance  
38  
39 profiles attributed to LPS-bilayers being squeezed out due to enhanced fluidity of the  
40  
41 LPS acyl-chain, with a final hard wall surface separation  $D_0 \sim 8\text{-}10$  nm corresponding  
42  
43 to the thickness of a single bilayer confined between the surfaces. These  
44  
45 unprecedented SFA results reveal intricate structural responses of LPS surface layers  
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3 to temperature and  $\text{Ca}^{2+}$ , with implications to our fundamental understanding of the  
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5 structures and interactions of bacterial membranes.  
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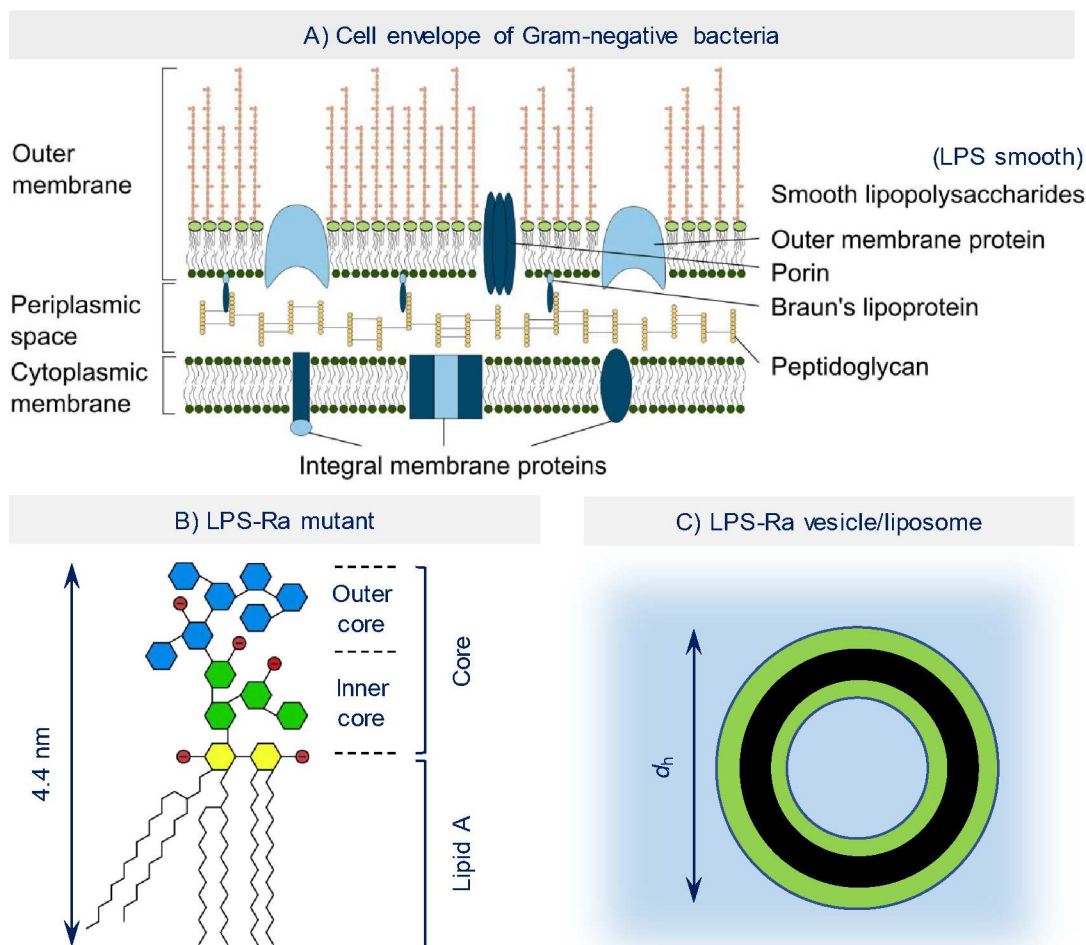
## 10 **Introduction**

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14 Antibiotic resistance has become an increasingly important issue due to excessive use  
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16 of antibiotics in agriculture and health industry<sup>1-3</sup>, whilst development of new antibiotics  
17  
18 has become stagnated. The bacterial cell wall can be a target for effective and  
19  
20 alternative antibacterial agents due to its accessibility and its significance in cell  
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22 communication, protection and regulation<sup>4-7</sup>. It is thus crucial to study the structure and  
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24 to understand fundamental interactions at the bacterial cell wall.  
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30 Bacteria can be broadly classified into Gram-positive and Gram-negative species  
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32 according to the staining properties due to their different cell wall structures, which are  
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34 distinct from those of eukaryotic cells. Both types of bacteria have an inner cytoplasmic  
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36 phospholipid membrane with a distal layer rich in peptidoglycan, which is significantly  
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38 thicker in Gram-positive bacteria (30-100 nm). Gram-negative bacteria possess an  
39  
40 additional highly asymmetric outer membrane, containing an inner phospholipid and  
41  
42 an outer bacteria-specific lipopolysaccharide (LPS) leaflet (Figure 1A), offering an  
43  
44 extra polymer brush-like protective coating<sup>4</sup>. Despite a high degree of structural  
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46 variability, some general features of LPS from different bacteria can be identified. It  
47  
48 consists of a conserved hydrophobic anchor, lipid A, and a polysaccharide head group,  
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50 which can be further divided into a moderately conserved, short core and a highly  
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3 variable, long O-antigen<sup>8</sup>. Mutant LPS with truncated head groups, ranging from free  
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5 lipid A to a full core (Ra mutant; Figure 1B) have been used as model systems to study  
6  
7 the influence of the carbohydrate chain architecture on LPS properties<sup>9, 10</sup>.  
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11 LPS not only serves as a protective coat, it can also trigger lethal septic shocks when  
12  
13 released into bloodstream in a molecular or aggregate form<sup>11, 12</sup>. Therefore, a number  
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15 of strategies in the combat against bacteria have targeted LPS. For instance, naturally  
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17 occurring cationic antimicrobial peptides (AMPs) exploit the presence of negatively  
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19 charged inner core and lipid A of LPS by binding electrostatically and subsequently  
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21 disrupting the membrane<sup>13-15</sup>. Naturally occurring and synthetic surface adsorbing  
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23 peptides and polymers have been reported to bind LPS specifically to remove LPS  
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25 and bacteria from solutions<sup>16-18</sup>. Other studies have reported LPS crosslinking induced  
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27 by cationic peptides and polymers, which can lead to the suppression of the endotoxic  
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29 effect<sup>19-21</sup>.  
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35 Figure 1. A) Schematic representation of the cell envelope of Gram-negative bacteria, showing the  
36 inner (cytoplasmic) and outer membranes and their main constituent molecules (including different  
37 proteins and the inter-linking peptidoglycan layer in the periplasmic space). Smooth  
38 lipopolysaccharides (LPS) are a major component of the outer leaflet of the outer membrane (Adapted  
39 from <sup>22</sup>). B) Schematic showing the structure of LPS-Ra mutant, with lipid A and inner and outer core  
40 regions. C) Possible LPS-Ra vesicle structure as suggested by the hydrodynamic diameter  $d_h$  from DLS  
41 measurement of the LPS-Ra aggregation in aqueous media, with the dark inner region representing  
42 LPS tails and the green regions LPS headgroups.

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51 Bacterial model membranes based on natural and mutant LPS have been prepared  
52 including monolayers at the air-water interface<sup>23-25</sup> and hydrophobized surfaces<sup>26, 27</sup>,

1 supported symmetric and asymmetric bilayers<sup>9, 28</sup>, and multilayers<sup>29</sup>. These model  
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3 membranes have been used to elucidate structural properties and interactions of  
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5 immobilised LPS monolayers with abiotic surfaces such as mica as well as  
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7 polystyrene- and APTES-coated mica<sup>27</sup>. Adsorption behaviour of LPS from aqueous  
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9 solution on abiotic surfaces has also been studied<sup>30-35</sup>. In addition, the effects of  
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11 different salts<sup>24</sup>, antimicrobial peptides<sup>23</sup>, and temperature<sup>29</sup> on these model  
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13 membranes have been studied experimentally and using computer simulations<sup>36-40</sup>.  
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21 Generally, LPS adsorption and interactions at surfaces have been considered as being  
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23 governed by H-bonding<sup>32</sup>, hydrophobic forces<sup>34</sup> and electrostatic forces<sup>27, 31, 35</sup>.  
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26 Despite these studies, there remain considerable gaps in our fundamental knowledge  
27  
28 on how physical parameters, such as the LPS carbohydrate chain length and  
29  
30 conformation, substrate surface physicochemical properties, and electrolytes, affect  
31  
32 the structure of the adsorbed LPS layers and the interactions at the LPS membrane  
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34 surface. Some consistencies and debates persisted in the literature. For instance,  
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36 Tong et al.<sup>31</sup> reported the formation of LPS-Rd and Ra bilayers in buffered solution on  
37  
38 mica, while Lu *et al.*<sup>27</sup> observed very little adsorption of LPS-Ra on the same surface.  
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40 In contrast, they found that *smooth LPS* adsorbed on mica and attributed it to hydrogen  
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42 (H-)bonding between the LPS and the surface; whereas little adsorption of *smooth*  
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44 *LPS* on hydrophilic (GeO<sub>2</sub>, Fe<sub>2</sub>O<sub>3</sub>, and Al<sub>2</sub>O<sub>3</sub>) and hydrophobic (ZnSe) surfaces was  
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46 observed by Parikh and Chorover<sup>34</sup>.  
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53 In comparison to monovalent cations, the presence of divalent cations can significantly  
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55 alter structural and interaction properties of LPS. Divalent cations can accumulate in  
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3 the negatively charged LPS core region<sup>9, 24</sup>, possibly crosslinking several lipid  
4 molecules<sup>41</sup> and inducing LPS multilamellar phases<sup>42</sup>. This can lead to LPS structural  
5 rearrangement, and associated membrane compaction can result in increased bilayer  
6 integrity<sup>9</sup> and thus reduced penetration by AMPs<sup>23</sup>. Divalent ions have also been  
7 shown to promote adsorption of LPS and bacteria<sup>43</sup> on negatively charged surface<sup>30</sup>.  
8 They have also shown strong effects on supramolecular aggregation of LPS in  
9 solution<sup>10, 44</sup>, which has been related to their interfacial adsorption behaviour<sup>34</sup>.

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21 In this study using the surface force apparatus (SFA)<sup>45, 46</sup>, we have investigated the  
22 adsorption behaviour of aggregates of LPS-Ra mutant (Figure 1, with its detailed  
23 chemical structure shown in Figure S1 in the Supplementary Information (SI) section)  
24 on negatively charge mica, and the interactions between these adsorbed layers; in  
25 particular, the effects of temperature and presence of Ca<sup>2+</sup> have been evaluated. The  
26 aggregate size has been characterised using DLS. Our results show that LPS Ra  
27 adsorption was significantly increased in the presence of Ca<sup>2+</sup>. In addition, the stability  
28 of the adsorbed layer altered as the temperature was raised above the gel to liquid  
29 crystalline ( $\beta$ - $\alpha$ ) acyl chain melting temperature of LPS-Ra ( $T_m = 36$  °C)<sup>10</sup>.  
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## 43 **Experimental**

### 44 ***Materials***

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47 Lipopolysaccharide (rough strain) from *Escherichia coli* EH100 Ra mutant (Sigma  
48 Aldrich®), calcium chloride (CaCl<sub>2</sub>; Acros Organics, ACS reagent grade) were used.  
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3 Ultrapure Milli-Q water (Resistivity: 18.2 M $\Omega$  cm, total organic content (ToC) < 4 ppb)  
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5 was used throughout for the solution preparation.  
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### 8 *Preparation and DLS/zeta potential characterisation of LPS-Ra aqueous dispersion*

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11 The extrusion method was used to homogenise the LPS-Ra aggregate size, as DLS  
12 suggested that highly polydisperse aggregates of size tens of nm – tens of  $\mu$ m existed  
13 in the non-extruded LPS-Ra solution. LPS-Ra (0.1 mg mL<sup>-1</sup>) in ultrapure Milli-Q water  
14 or in 2.5 mM aqueous CaCl<sub>2</sub> solution was vortexed for 30 sec before extrusion using  
15 a 10 mL LIPEX extruder (Thermobarrel, Northern Lipids Inc.) in combination with a  
16 temperature controlled water bath (Grant Scientific, Optima T100 + TC120) at a  
17 pressure ~ 5 bar. The solution was first extruded six times through two polycarbonate  
18 membranes (Whatman Nuclepore Track-Etch polycarbonate membrane, diameter 25  
19 mm) with a pore size of 0.2  $\mu$ m, and then further six times through two polycarbonate  
20 membranes of pore size 0.1  $\mu$ m, all at 80 °C to obtain a transparent solution. The  
21 hydrodynamic diameter ( $d_h$ ) and zeta potential ( $\zeta$ ) of the resulting solutions were  
22 obtained using dynamic light scattering DLS (Nano Zetasizer ZS, Malvern  
23 Instruments). Approximately 1 mL of the extruded solutions was injected into a plastic  
24 cuvette (Fisher, UV Grade Cuvettes) and the DLS measurements of  $d_h$  were taken,  
25 with the analysis performed using Zetasizer Software 7.12. Electrophoretic mobility  
26 ( $\mu_e$ ) measurement was made using disposable folded capillary cells (Malvern  
27 Instruments, DTS1070), with the sample left to equilibrate for at least 2 min prior to the  
28 measurement. The obtained  $\mu_e$  was converted to  $\zeta$  potential using the approach  
29 described by Delgado et al.<sup>47</sup>, which has its limitations when the  $\zeta$  value is greater than  
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3  $\pm 50$  mV<sup>48</sup>. We will have performed comprehensive  $\zeta$  measurements of LPS mutant  
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5 aggregates in solutions of monovalent and multivalent ions, which will be discussed in  
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7 a separate publication (Redeker et al., in preparation).  
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### 10 *Atomic force microscopy (AFM) imaging of morphology of surface layers*

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14 AFM (Bruker Multimode 8, Peakforce feedback control) was used to image the  
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16 morphology of LPS-Ra aggregates absorbed on mica. A  $\sim 1$  cm x 1 cm mica sheet was  
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18 glued onto an AFM magnetic disk using Epon 1004 (Shell) and subsequently the top  
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20 layers of mica removed using a piece of sticky tape to expose a clean, molecularly  
21  
22 smooth surface. The disk was incubated overnight in approximately 30 mL of extruded  
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24 LPS ( $0.1$  mg mL<sup>-1</sup>; equivalent of  $24.6$   $\mu$ M) aqueous dispersion (either in MilliQ water  
25  
26 or  $2.5$  mM CaCl<sub>2</sub> solution) at  $21$  °C prepared as described above. The AFM disk was  
27  
28 then transferred to the instrument with a drop of the solution on top to ensure  
29  
30 continuous wetting of the surface. A liquid cell containing aqueous CaCl<sub>2</sub> solution of  
31  
32 the same concentration as the sample under investigation was mounted above the  
33  
34 surface to prevent the surface from drying out during the measurement. Bruker  
35  
36 Scanasyst silicon nitride cantilevers were used for imaging (spring constant  $0.7$  N m<sup>-1</sup>,  
37  
38 tip radius  $2$ - $5$  nm). Images were obtained in the tapping and Quantitative  
39  
40 NanoMechanical (QNM) mode. Scan rates between  $0.2$ - $3$  Hz were used with image  
41  
42 sizes ranging from  $0.1$ - $10$   $\mu$ m. Images were recorded and processed, including  
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44 flattening to account for sample tilting and low pass filters to smoothen the image,  
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46 using the Bruker NanoScope Analysis 1.80 software package. To investigate the effect  
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48 of temperature on the adsorption and morphology of LPS-Ra aggregates on mica  
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3 (Islas et al. in preparation), disks were incubated in 30 mL LPS-Ra solution at 45 °C  
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5 for 1-12 h. Subsequently, the solution containing the disks were left to cool to room  
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7 temperature and then imaged as described above at room temperature.  
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### 10 11 ***Measurements using the Surface force apparatus (SFA)*** 12

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14 The SFA<sup>45, 46, 49</sup> was used to obtain normal ( $F_N$ ) and shear ( $F_s$ ) forces between two  
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16 mica surfaces immersed in aqueous LPS dispersions as a function of surface  
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18 separation ( $D$ ). Mica (S & J Trading Inc., A1 special grade) was cleaved by hand in a  
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20 particle-free laminar flow hood (LFH) to 2-7  $\mu\text{m}$  thick sheets, which were then cut into  
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22  $\sim 1 \times 1$  cm pieces using a white-hot platinum (Pt) wire (99.9999% pure; Sigma), and  
23  
24 immediately laid down on a freshly cleaved mica backing sheet to protect them from  
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26 dust. Care was taken during the cutting so that the mica sheets “upstream” in the LFH  
27  
28 from the Pt wire, so that they were not exposed to any possible nanoparticles  
29  
30 generated by the hot wire. A thin layer ( $\sim 40$  nm) of silver (Ag beads, Aldrich, purity:  
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32 99.9999%) was deposited onto the mica using thermal evaporation under  $\sim 10^{-6}$  Torr  
33  
34 (Edwards Coating System E306A). The mica sheets were then glued onto cylindrical  
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36 quartz disks using Epon 1004 (Shell) with the silvered side down, and the disks were  
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38 mounted in a crossed cylinder configuration.  
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3 Instruments) with  $\sim 1.5$  nm resolution. The lower surface is also mounted on a pair of  
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5 leaf springs ( $k_N = 100$  N m<sup>-1</sup>) in a liquid cell, which is attached to a linear stepping  
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7 motor (Physik Instrumente (PI) GmbH & Co. KG) within a motion range of 20 mm, a  
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9 step size  $<1$  nm, and a speed range between 1 and  $10^5$  nm s<sup>-1</sup>. The upper surface  
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11 can be moved parallel to the lower surface by the application of an oscillatory voltage  
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13 of designated amplitude and frequency (e.g. a sawtooth function with a peak to peak  
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15 amplitude of 3  $\mu$ m) to the piezoelectric tube. Digital signal traffic control and monitoring  
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17 input and output is managed using a field programmable gate array (FPGA) via a  
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19 LabView interface programme.  
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26 Surface separation  $D$  in the SFA is monitored using fringes of chromatic order (FECO)  
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28 based on multiple beam interferometry in the optical cavity of silvered mica surfaces,  
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30 by shining a beam of collimated white light generated by an arc lamp (Newport  
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32 Spectra-Physics Ltd.). The FECO fringes are resolved in a Shamrock 500  
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34 spectrometer with a Neo sCMOS camera at speeds of up to 100 frames per second.  
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36 Images of FECO during approach and separation of the surfaces are recorded with  
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38 the time-stamped motor position. The digital FECO spectral images can be converted  
39  
40 into surface separations using the transfer matrix method<sup>50</sup>. The force acting between  
41  
42 the two surfaces at any given separation  $D$  can be calculated as  $F_N(D) = k_N \Delta D$  where  
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44  $\Delta D$  is the deflection of the horizontal springs, i.e.  $\Delta D = D - D_0$  with  $D_0$  extrapolated  
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46 from the linear surface separation vs. motor position plot at large  $D$  where the force is  
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48 absent. Similarly, the shear force  $F_s$  can be determined from the deflection  $\Delta x$  of the  
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50 shear springs measured with the capacitance probe, i.e.  $F_s(D) = k_s \Delta x$ .  
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3 Approximately 18 mL of the LPS dispersion was injected between the two surfaces in  
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5 a specific sequence (LPS-Ra in water, LPS-Ra in 2.5 mM aqueous CaCl<sub>2</sub> solution and  
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7 pure water) and normal and shear forces between the surfaces measured in each  
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9 solution at a temperature below (21 °C) and above (40 °C) the phase transition  
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11 temperature of LPS Ra (36 °C<sup>10</sup>). The SFA results below represent data collected  
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13 from 20 different contact spots from 5 separate experiments.  
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## 22 **Results and discussion**

### 23 *DLS of LPS-Ra aggregates and AFM imaging of LPS-Ra surface layers*

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28 Figure 3A shows that the DLS hydrodynamic diameter  $d_h$  of the extruded LPS-Ra  
29  
30 dispersion increased from  $28 \pm 5$  nm in pure water to ~70-90 nm in 5-10 mM CaCl<sub>2</sub> at  
31  
32 room temperature (~21 °C), and the corresponding zeta potential  $\zeta$  became less  
33  
34 negative concomitantly. Such a self-association behaviour in solution is largely  
35  
36 consistent with previous DLS studies which reported aggregate hydrodynamic  
37  
38 diameters ranging from 14-95 nm for different rough and smooth LPS<sup>51-54</sup> and  $\zeta$  ~-30  
39  
40 mV for LPS-Ra in HEPES buffered solution (pH 7)<sup>55</sup>. Given the molecular length of the  
41  
42 LPS-Ra of 4.4 nm (Figure 1B), we postulate that they formed vesicle-like structures in  
43  
44 both pure water and the CaCl<sub>2</sub> solution (also see AFM images in Figure 4 below).  
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46 Cryogenic transmission electron microscopy (CryoTEM) has previously revealed a  
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48 variety of structures of unextruded LPS aggregates<sup>56, 57</sup> ranging from spherical, to  
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50 fibrillar and toroidal structures depending on the presence of electrolytes and  
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3 carbohydrate chain length. In particular, for non-extruded LPS in HEPES buffer,  
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5 CryoTEM showed vesicular structures for lipid A (diameter range from 100 nm to 3  
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7  $\mu\text{m}$ ), LPS-Re (diameter range from  $<100$  nm to  $\sim 250$  nm) and LPS-Rc (diameter range  
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9 from 250 nm to 500 nm) from *Salmonella enterica* sv. Minnesota; while non-extruded  
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11 LPS-Ra in HEPES buffer from the same bacterial species showed predominately  
12  
13 ribbon-like bilamellar structures (of greater than some hundred nanometers in  
14  
15 length)<sup>56</sup>. These observations of vesicular aggregate formation for LPS mutants are in  
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17 agreement with our postulated aggregate structure, although Richter et al.<sup>56</sup> showed  
18  
19 predominately ribbon-like bilamellar structures for LPS-Ra. Such a discrepancy may  
20  
21 be due to different solution conditions (i.e. LPS-Ra concentration and pH value) and  
22  
23 the bacterial origin of LPS-Ra used by Richter et al.<sup>56</sup> compared to our study.  
24  
25 Furthermore, it is conceivable that the long ribbon-like bilamellar structures of Richter  
26  
27 et al.<sup>56</sup> may be transformed into vesicles by the extrusion process. Finally, freezing  
28  
29 LPS-Ra vesicles for CryoTEM sample preparation could have also led to the collapse  
30  
31 of vesicular LPS-Ra structures into the observed ribbon-like bilamellar structures. It  
32  
33 should be acknowledged that It is extremely challenging to characterise the LPS  
34  
35 aggregate structures using classic neutron and X-ray scattering methods (which we  
36  
37 are currently pursuing) due to the size and structural complexity/diversity. In this study,  
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39 we have homogenised our samples *via* extrusion to reduce the structural complexity  
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41 and polydispersity, as required by SFA measurements.  
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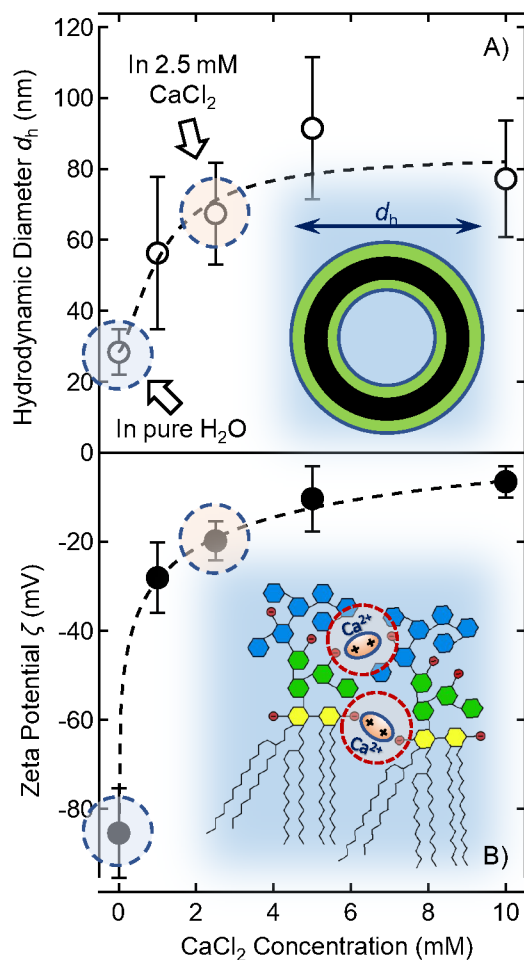


Figure 3. A) Hydrodynamic diameter  $d_h$  of LPS-Ra aggregates as a function of CaCl<sub>2</sub> concentration at 21 °C, measured using the dynamic light scattering (DLS) method. The inset schematic shows the postulated vesicle aggregate structure. B) Zeta potential  $\zeta$  vs. CaCl<sub>2</sub> concentration. The dashed lines are a guide to the eye, and the dark-blue dashed-circles indicate data points in pure water and 2.5 mM CaCl<sub>2</sub>, respectively, conditions under which SFA measurements have been performed. The inset schematic shows Ca<sup>2+</sup> bridging (red dashed-circles) between negatively charged phosphate groups in the adjacent lipid A headgroup and inner/outer core regions of Lipid-Ra.

Divalent cations have been reported to accumulate in the negatively charged phosphate groups in the LPS core and the lipid A headgroup region (cf. Figure 3B inset)<sup>9, 24</sup>, and the enhanced inter-molecular binding could conceivably enhance the effective membrane rigidity, leading to a decrease in the aggregate curvature and thus

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2  
3 an increase in  $d_h$ , as  $\text{Ca}^{2+}$  concentration increased. The interpretation of increased  
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5  $\text{Ca}^{2+}$  adsorption to LPS-Ra aggregates with the  $\text{CaCl}_2$  concentration is also consistent  
6  
7 the  $\zeta$  potential data shown in Figure 3B, with  $\zeta$  decreasing from  $\sim -85 \pm 10$  mV in pure  
8  
9 water to  $-10 \pm 5$  mV due to cation adsorption. In contrast, the aggregate  $d_h$  values  
10  
11 remained largely constant at  $\sim 28$  nm in 1-10 mM NaCl (data not shown; Redeker et  
12  
13 al., ms in preparation). The dark-blue dashed circles in Figure 3 indicate the data  
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15 points for the solution conditions (pure water and 2.5 mM  $\text{CaCl}_2$ ) in which the SFA  
16  
17 measurements have been performed, i.e. the aggregate size  $d_h = 28 \pm 5$  nm and  $68 \pm$   
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19  $13$  nm and the corresponding zeta potential  $\zeta = -85 \pm 10$  mV and  $-20 \pm 5$  mV in pure  
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21 water and 2.5 mM  $\text{CaCl}_2$ , respectively.  
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28 Figure 4 shows representative AFM height images of mica immersed in the extruded  
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30  $0.1 \text{ mg mL}^{-1}$  LPS-Ra dispersions in water and 2.5 mM  $\text{CaCl}_2$  at 21 °C, with the imaging  
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32 undertaken after the mica surface was incubated in the dispersion at  $\sim 45^\circ\text{C}$ , above  
33  
34 the melting temperature  $T_m = 36$  °C of the lipid tails in LPS-Ra, and then cooled down  
35  
36 to 21 °C. Little adsorption of any LPS-Ra aggregates as observed in water (Figure 4A;  
37  
38 corresponding 3D image shown in Figure S2 in SI), with some small (a few nm in the  
39  
40 lateral dimension) surface features that could be attributed to small patches of  
41  
42 adsorbed LPS-Ra – possibly aggregate fragments, consistent with the surface force  
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44 curve ② in Figure 5 discussed below. In contrast, 2D height image in Figure 4B and  
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46 3D image in Figure 4C show the presence of the adsorbed surface layers in 2.5 mM  
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48  $\text{CaCl}_2$ , with the surface layer exhibiting spherical or ellipsoidal shapes of different sizes  
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50 ( $\sim 50$ -250 nm) and heights (50-100 nm, cf. the line profile in Figure 4B). We attribute  
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3 the observed surface structures to adsorbed LPS-Ra aggregates, probably with loose  
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5 distal layer(s) atop an underlayer, and our initial analysis (Islas et al., in preparation)  
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7 of the lateral size of the surface aggregates is consistent with them deformed/flattened  
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9 laterally. The morphology (i.e. spherical-like) is also broadly consistent with the DLS  
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11  $d_h$  results (Figure 3A), substantiating in part the postulation that LPS-Ra vesicles  
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13 formed in solution. Further experimental evidence, e.g. using small-angle neutron  
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15 scattering (SANS), could provide corroborating evidence for vesicle formation.  
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18 However, our preliminary SANS experiment showed weak scattering from the  
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20 aggregates in in the LPS-Ra concentration range in which these aggregates were  
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25 stable.  
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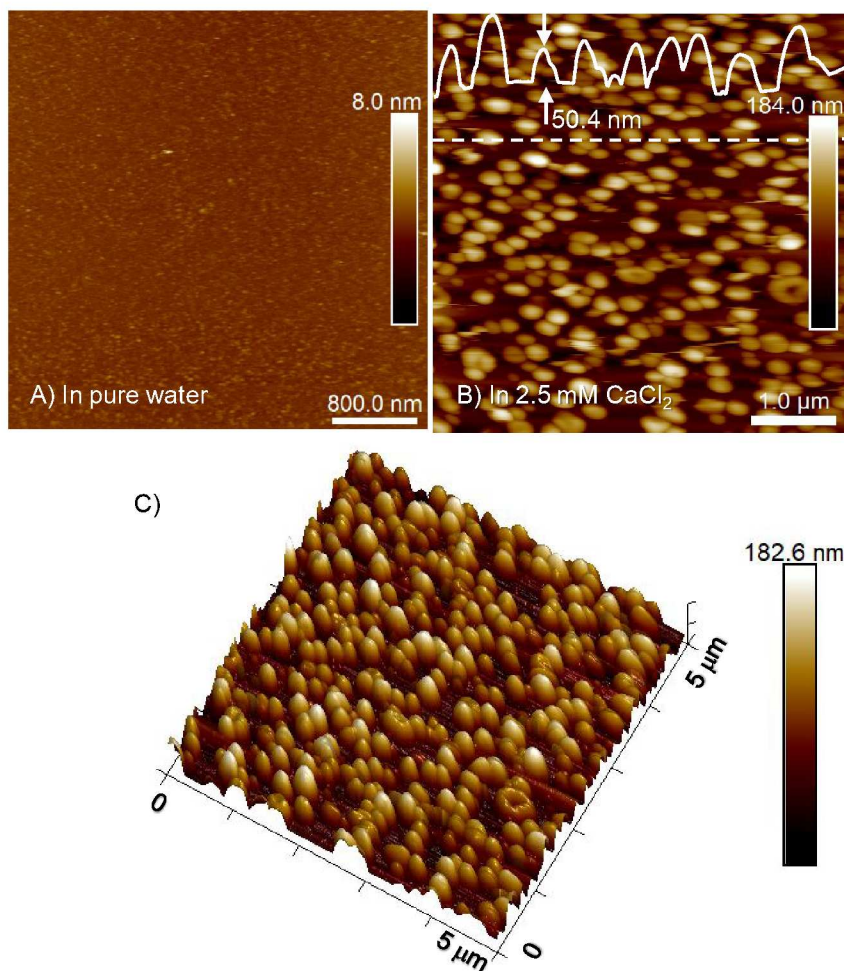


Figure 4. (A) AFM height image ( $4\ \mu\text{m} \times 4\ \mu\text{m}$ ) of mica immersed in  $0.1\ \text{mg mL}^{-1}$  LPS-Ra dispersions in pure water, showing little surface adsorption. The corresponding 3D image is shown in Figure S2 in the SI section. (B) AFM height image ( $5\ \mu\text{m} \times 5\ \mu\text{m}$ ), with the corresponding 3D image (C), of mica immersed in  $0.1\ \text{mg mL}^{-1}$  LPS-Ra dispersion in  $2.5\ \text{mM CaCl}_2$ , showing adsorbed layers of aggregates. The imaging was undertaken after the surfaces were incubated in the respective dispersion at  $\sim 45\ ^\circ\text{C}$  and then cooled down to  $21\ ^\circ\text{C}$ . An example cross section line profile along the white dashed-line in the figure reveals surface aggregates of different heights (50-100 nm, with one of them indicated) and widths ( $\sim 100$ - $150\ \text{nm}$ ), attributed to the adsorbed flattened LPS-Ra vesicles, possibly with additional adsorption atop an underlying layer.

Nonetheless, it is clear from Figure 4 that  $\text{Ca}^{2+}$  has a significant effect on the adsorption behaviour of the extruded LPS-Ra dispersion on mica. In pure water, the repulsion between the negatively charged LPS-Ra core and lipid A headgroup and

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3 negatively charged mica surface sites vacated by desorption of lattice  $K^+$  would  
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5 suppress any significant LPS-Ra adsorption. In 2.5 mM  $CaCl_2$ , inter-LPS bridging by  
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7  $Ca^{2+}$  would have enhanced the stability of the vesicles against spontaneous fusion  
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9 and bilayer formation on the surface observed with liposomes<sup>58</sup>. Similar trends of  
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11 increased resistance to rupture upon  $Ca^{2+}$  addition have been reported for supported  
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13 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and DMPC bilayers<sup>59</sup>.  
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15 Concurrently, divalent  $Ca^{2+}$  could bridge the similarly charged lipid A heagroups and  
16  
17 mica surface sites (cf. Figure 6), in a manner not unlike the intramolecular bridging  
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19 effect that  $Ca^{2+}$  exhibits between negatively charged LPS-Ra phosphate groups.  
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21 Enhanced LPS-Ra vesicle stability and augmented LPS-mica binding mediated by  
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23  $Ca^{2+}$  would have facilitated the observation of the relatively stable surface vesicle layer  
24  
25 by AFM imaging in Figure 4B,C. The morphological details of LPS-Ra aggregates  
26  
27 adsorbed on mica depended on  $Ca^{2+}$  concentration and solution temperature, effects  
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29 currently under investigation (Redeker et al. ms in preparation).  
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### ***Interactions between mica surfaces immersed in LPS-Ra dispersions in pure water***

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41 Figure 5A shows example profiles (Curve ①) of the normal force normalised by the  
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43 effective surface radius vs. surface separation ( $F_N/R$  vs.  $D$ ) between two mica surfaces  
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45 immersed in the extruded LPS-Ra (0.1 mg/mL) dispersions in pure water at 21 °C,  
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47 both upon approach (empty circles) and retraction (filled circles) between the two  
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49 surfaces. In pure water, a relatively long-range repulsion (diminishing at  $\sim D = 60 - 80$   
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51 nm) was observed, before the surfaces reached a hard-wall repulsion at  $D_0 = 0 - 3$  nm  
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3 (which varied from different contact spots). No adhesion was observed upon  
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5 retraction, with a small hysteresis compared to the approach.  
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9 We attribute the observed long-range repulsion – exponential in nature (*cf.* inset to  
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11 Figure 5A) – to the electrical double layer repulsion arising from the residual surface  
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13 charge after patchy LPS-Ra adsorption (*cf.* Figure 4A), which would also account for  
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15 the short-range repulsion and the hysteresis between the approach and retraction  
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17 force profiles due to deformation in the patchy layer occurring upon compression and  
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19 subsequent relaxation upon retraction. The effective decay length of  $\kappa^{-1} = \sim 18\text{-}20$  nm  
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21 is much smaller than that in pure water ( $\sim 96$  nm), indicating that LPS-Ra contributed  
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23 to the background ionic strength and screening. *E. coli* LPS-Ra EH100 carries 3  
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25 phosphates, a single phosphoethanolamine (PEtN) and a single  
26  
27 pyrophosphoethanolamine (PPEtN) as well as 2 carboxylic groups, thus with a  
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29 maximum of 11 ionizable groups<sup>60</sup>. Calculation of the Debye length  $\kappa^{-1}$  using the LPS-  
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31 Ra concentration of 0.1 mg/mL (24.6  $\mu\text{M}$ ) and assuming full dissociation of the charged  
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33 groups and a highly asymmetric (1:11 anion:cation) electrolyte, would yield  $\kappa^{-1} = 7.54$   
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35 nm, much smaller than the fitted decay length. For to obtain  $\kappa^{-1} = \sim 18\text{-}20$  nm, we  
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37 would need to assume 3.85-4.34 (or 35-40%) of the 11 groups were charged.  
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39 Furthermore, aggregation of LPS into different shapes and sizes<sup>56, 57</sup> indicates the  
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41 presence of an apparent critical aggregation concentration (CAC) which is dependent  
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43 on the LPS polysaccharide chain length, although no CAC value has been reported  
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45 for LPS-Ra in the literature. The CAC for short polysaccharide chain mutant LPS-Re  
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47 and Lipid A from *S. minnesota* were 4  $\mu\text{M}$  and 5  $\mu\text{M}$  respectively<sup>53</sup>, although the validity  
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3 has been questioned due to experimental limitations<sup>61</sup>. In contrast, Mueller et al.<sup>12, 62</sup>  
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5 estimated a CAC of ~10 nM for these LPS, while Sasaki et al.<sup>63</sup> found a temperature  
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7 dependency for LPS-Re:  $T < T_m$ : 41.2 nM;  $T > T_m$ : 8.1 nM). The CACs for LPS from  
8  
9 heterogenous long O-antigen polysaccharide chain bacterial serotypes ranged from  
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11 10  $\mu\text{g/mL}$  to 32  $\mu\text{g/mL}$ <sup>51, 53, 54</sup>, depending on the bacterial type. For LPS from  
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13 *Escherichia coli* serotype 026:B6, a CAC of 14  $\mu\text{g/mL}$  (3.2  $\mu\text{M}$ ) was reported in the  
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15 PBS buffer. For our measurements with LPS in pure water, if only the background  
16  
17 (CAC) LPS concentration was used, it would yield a  $\kappa^{-1}$  ~20-30 nm, compared to the  
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19 fitted value of  $\kappa^{-1} = 18\text{-}20$  nm. Hence, it appears that the charged aggregates and their  
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21 dissociated counterions might have contributed to the screening in the measured  
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23 electric double layer force.  
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31 A short-range repulsion onset at  $D \sim 3\text{-}5$  nm ( $F_N/R \sim 40$  mN m<sup>-1</sup>), then increased sharply  
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33 as the surfaces were pushed closer into contact, attributed to adsorbed LPS on the  
34  
35 surface. This hard wall contact separation,  $D_0 \sim 0\text{-}2.8$  nm, varied between different  
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37 contact spots and different experiments, indicating inhomogeneous surface coverage.  
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39 In the particular force Curve ① shown in Figure 5, there is also evidence of the surface  
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41 layer being squeezed out from  $D \sim 1.2$  nm to  $D \sim 0.2$  nm, as indicated by the slight  
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43 discontinuity in the force profile. If we assume symmetric surface adsorption, a surface  
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45 layer thickness of  $t \sim 0.6\text{-}2.5$  nm formed but the distribution was not uniform. At other  
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47 contact spots, the surfaces could be push to  $D_0 \sim 0$  nm contact, and also there was a  
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49 slight hysteresis in the force profile as the surfaces were separated (filled circles in the  
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51 figure). This suggests that at these spots the adsorbed layer could be completely  
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3 removed or there was little adsorption - the surface coverage was patchy due to weak  
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5 adsorption of LPS-Ra (or its vesicle fragments) on the surface. This is likely due to  
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7 strong electrostatic repulsion between negatively charged mica and LPS-Ra in  
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9 solution as indicated by a measured zeta potential  $\zeta = -56.7$  mV at 25°C for LPS-Ra  
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11 dispersion in pure water (cf. Figure 3B). The surface layer thickness is also  
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13 comparable to that detected from SFA experiments by Lu et al.<sup>27</sup>, who used a similar  
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15 LPS-Ra mutant but from a different strain of *Escherichia coli* (K12) in 0.1 M NaCl  
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17 solution on mica, observing the formation of surface layers of  $t \sim 1$  nm in thickness. In  
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19 contrast to our results, they found a small adhesion force ( $-0.7$  mN m<sup>-1</sup>) upon retraction.  
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22 However, overall the presence of a high concentration of potassium cations seemed  
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28 to have little effect on the LPS adsorption behaviour.  
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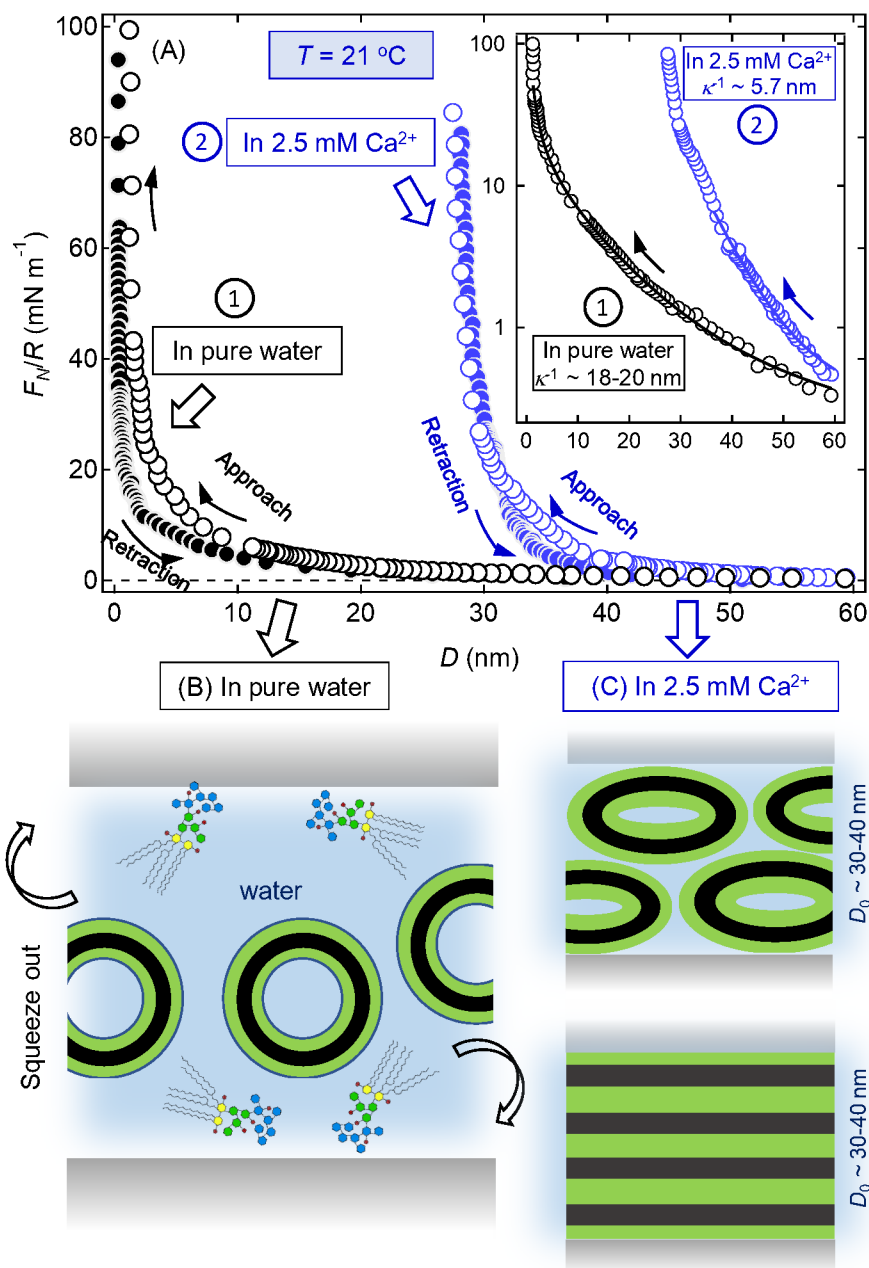


Figure 5. (A) Normal surface force normalised by the contact radius vs. surface separation ( $F_N$  vs.  $D$ ) between mica immersed in  $0.1 \text{ mg mL}^{-1}$  LPS-Ra dispersions in pure water (Curve ①) and 2.5 mM  $\text{CaCl}_2$  (Curve ②) at  $21^\circ\text{C}$  upon approach (empty circles) and retraction (filled circles) of the surfaces. The inset shows the corresponding log-linear plot for the approach force profiles, with the solid curves showing the exponential fit to the long-range decay of the force profiles, with the effective Debye length also given ( $\kappa^{-1} = 18\text{-}20$  nm in water and  $\kappa^{-1} = 5.7$  nm in 2.5 mM  $\text{CaCl}_2$ , respectively). (B) Schematic showing LPS-Ra vesicles being squeezed out in pure water. (C) Schematic showing two possible surface structures giving rise to the surface force profile ② in 2.5 mM  $\text{CaCl}_2$ : surface-

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3 adsorbed flattened LPS-Ra vesicles (top) and multilayers of LPS-Ra membranes due to rupturing of  
4 vesicles on the surface. The bridging role of  $\text{Ca}^{2+}$  is further illustrated in Figure 6.  
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7 The surface force profiles were not significantly affected by increased incubation time  
8 (up to 1 day) of the surfaces in the LPS solution. Furthermore, increasing the  
9 temperature in pure water to above the LPS-Ra  $\beta$ - $\alpha$  acyl chain melting temperature  
10 ( $T_m = 36^\circ\text{C}$ ) had little effect on the surface force profiles, except for a small increase  
11 ( $\delta t < 1$  nm) in the surface layer thickness. This could be attributed to slightly increased  
12 adsorption because of reduced electrostatic repulsion due to screening, consistent  
13 with a slight decrease in absolute zeta potential value of LPS-Ra dispersion in pure  
14 water from  $\zeta = -56.7$  mV at  $25^\circ\text{C}$  to  $\zeta = -52.5$  mV at  $40^\circ\text{C}$ .  
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27 ***Interactions in LPS-Ra dispersions in 2.5 mM  $\text{CaCl}_2$  at  $21^\circ\text{C}$  (below the melting***  
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29 ***temperature  $T_m = 36^\circ\text{C}$  of LPS-Ra acyl chains)***  
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32 The addition of 2.5 mM  $\text{CaCl}_2$  had a dramatic effect on the adsorption behaviour of  
33 LPS-Ra on mica.  $F_N/R$  vs.  $D$  profiles labelled ② in Figure 5A shows example surface  
34 force curves between two mica surfaces immersed in the extruded LPS-Ra (0.1  
35 mg/mL) dispersion in 2.5 mM  $\text{CaCl}_2$  at  $21^\circ\text{C}$  after overnight incubation, upon approach  
36 (empty circles) and retraction (filled circles) between the two surfaces. A purely  
37 repulsive force was observed when the surfaces were brought into contact. However,  
38 the hard wall thickness increased to  $D_0 = 30\text{-}40$  nm in 2.5 mM  $\text{CaCl}_2$  compared to  $D_0$   
39  $= 0\text{-}2.8$  nm in pure water (Curve ① in Figure 5A). Given the LPS-Ra bilayer thickness  
40  $\delta D \sim 8\text{-}10$  nm,  $D_0 = 30\text{-}40$  nm could be accounted for by  $\sim 4$  LPS-Ra bilayers, either  
41 due to a layer of flattened LPS-Ra vesicles on each surface or due to trapped 4  
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3 bilayers from compression-induced vesicle rupture (Figure 5C). These two scenarios  
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5 could not be distinguished unequivocally from the force profiles at 21 °C, and it is likely  
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7 that both could occur. However, as we discuss below, our experimental observations  
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9 point to trapped multilayers between the surfaces under elevated temperatures ( $T >$   
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11  $T_m$ ). There is a slight hysteresis in the force profile upon retraction, attributed to the  
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13 relaxation of the compressed surface layer. The details of the surface profiles varied  
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15 at contact spots, and another example of the  $F_N/R$  vs.  $D$  profiles is shown in Figure 6A  
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17 below (Curve ①) with a more noticeable hysteresis upon retraction. However, the  
18  
19 overall consistent feature of the surface force profiles in 2.5 mM  $\text{CaCl}_2$  is a purely  
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21 repulsive interaction, reaching a hard wall at  $D_0 = 30\text{-}40$  nm.  
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28 Preceding to reaching the hard wall, a short-range repulsion with an apparent  
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30 exponential decay length of  $\kappa^{-1} = 5\text{-}6$  nm was observed (cf. Figure 5A inset; Curve ②).  
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32 The calculated Debye length in 2.5 mM  $\text{CaCl}_2$  (overwhelming the LPS-Ra  
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34 concentration of 24.6  $\mu\text{M}$ ) is  $\kappa^{-1} = 3.5$  nm. An effective background  $\text{CaCl}_2$  concentration  
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36 of 1 mM would have been needed to yield  $\kappa^{-1} = 5\text{-}6$  nm, and adsorption of  $\text{Ca}^{2+}$  could  
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38 not have been sufficient to account for the depletion of  $\text{Ca}^{2+}$  concentration. One  
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40 possibility is that the presence of LPS-Ra might have suppressed dissociation of  
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42  $\text{CaCl}_2$ . Alternatively, the repulsion is not solely of an electrical double layer origin;  
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44 instead, it could be due to elastic deformation of the surface adsorbed LPS-Ra layers,  
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46 or that of a loosely adsorbed top layer (either vesicles or their fragments) atop the  
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48 surface layer. We are currently developing an analytic model to possibly relate this  
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50 short-range repulsion to the elastic modulus of the surface adsorbed layer. However,  
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3 the overriding feature of our observation here is the dramatic effect of  $\text{Ca}^{2+}$  on the  
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5 adsorption of LPS-Ra layers on mica.  
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9  $\text{Ca}^{2+}$  is known to bind to the negatively charged phosphate groups in the LPS core and  
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11 the lipid A headgroup region <sup>9, 24</sup>, which is also consistent with our zeta potential data  
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13 (cf. Figure 3B). Lanne et al. <sup>64</sup> have suggested that phosphate groups may also be  
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15 present in the outer core of LPS (cf. Figure 1B), and thus  $\text{Ca}^{2+}$  could also bind to LPS-  
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17 Ra outer cores. Accordingly, in the schematic of Figure 6 (and also the inset in Figure  
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19 Ra outer cores. Accordingly, in the schematic of Figure 6 (and also the inset in Figure  
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21 3B), the bridging effect of  $\text{Ca}^{2+}$  between LPS-Ra is shown to be mediated between the  
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23 negatively charged lipid A headgroups and between the inner and outer cores of LPS-  
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25 Ra. Furthermore, the enhanced adsorption of LPS-Ra aggregates in 2.5 mM  $\text{CaCl}_2$  –  
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27 evident from both the AFM imaging (Figure 4B,C) and the surface force profiles (Curve  
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29 ② in Figure 5A) – can be attributed to the bridging effect mediated by  $\text{Ca}^{2+}$  between  
30  
31 the negatively charged phosphate groups in the outer and inner cores of LPS-Ra and  
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33 the negatively charged mica sites residual from desorption of  $\text{K}^+$  from the lattice.  
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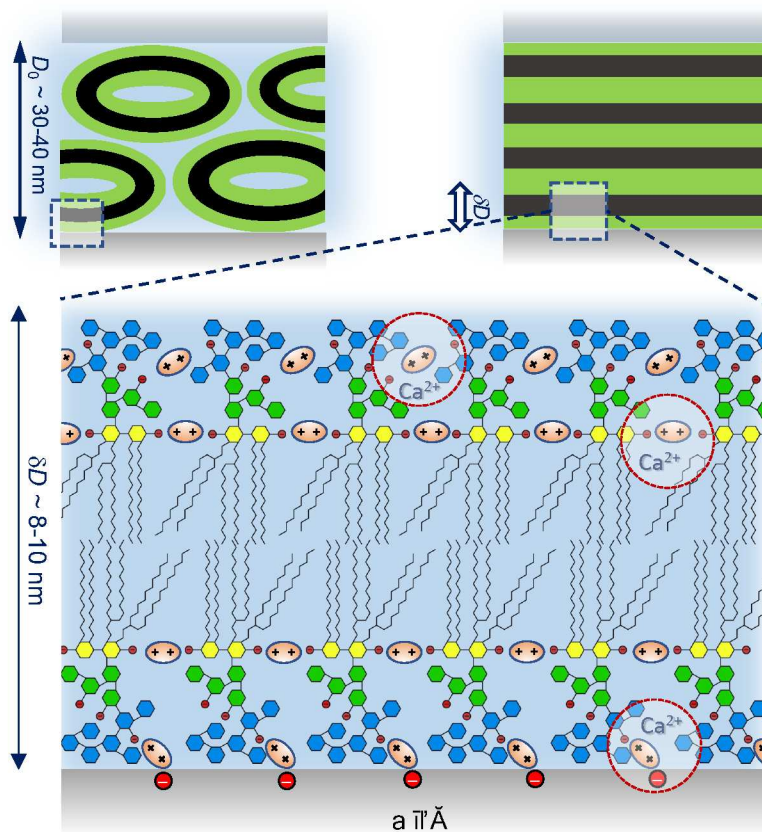


Figure 6. Schematic showing an LPS-Ra bilayer of thickness  $\delta D \sim 8-10$  nm adjacent to negatively charged mica in  $\text{CaCl}_2$  solution, applicable to the both scenarios of LPS-Ra vesicles or multilayers at the mica surface. The bridging effect of  $\text{Ca}^{2+}$  is schematically shown between the negatively charged phosphate groups in the inner and outer core regions of LPS-Ra, and between the negatively charged outer core and the negatively charged mica sites.

### ***Interactions in LPS-Ra dispersions in 2.5 mM $\text{CaCl}_2$ at 40 °C ( $T > T_m$ )***

The effect of temperature on the LPS-Ra surface layers was investigated by SFA measurements at temperatures above the  $\beta$ - $\alpha$  acyl chain melting temperature of LPS-Ra ( $T_m = 36$  °C). At 21 °C (Curve ① in Figure 7A), a hard wall was reached at  $D_0 = 30-40$  nm under a compressive force  $F_N/R$  up to 700-800  $\text{mN m}^{-1}$  (also see Figure 5A). In contrast at 40 °C, an example force profile in Figure 7A (Curve ②; contact spot 1) shows that the surfaces encountered the first hard wall at  $D_{0,1} = 27$  nm, before an

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3 inward step of size  $\delta D \sim 9$  nm at  $F_N/R \sim 200$  mN m<sup>-1</sup> to a second hard wall contact  $D_{0,2}$   
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5 = 18 nm. We attribute the observed force profiles at 40 °C to arising from 3-4 LPS-Ra  
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7 bilayers confined between the surfaces. The force profile ② on a log-linear scale in  
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9 the inset in Figure 7A reveals a repulsion preceding the hard wall contact at  $D_{0,1}$ , which  
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11 could be attributed to the removal of the loosely attached top LPS-Ra top layer.  
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16 As AFM images in Figure 4B,C revealed surface vesicles of LPS-Ra would have  
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18 formed on a single mica surface. As the surfaces were brought close to each other,  
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20 the confinement would encourage vesicle rupture to form bilayers, a process further  
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22 promoted by the enhanced acyl tail fluidity at  $T > T_m$ . Spontaneous rupture of  
23  
24 liposomes or vesicles is a classic method for lipid bilayer formation, which would often  
25  
26 lead to single bilayers on a solid substrate (e.g. <sup>58</sup>). The LPS-Ra lipids, with their quite  
27  
28 unique molecular architecture, would sustain structural integrity of the vesicles,  
29  
30 resisting surface rupture and forming layers of surface vesicles as observed from AFM  
31  
32 imaging (cf. Figure 4B,C). The fact that more than 3-4 bilayers were observed between  
33  
34 the surfaces points to confinement induced rupture, which would have left the bilayers  
35  
36 trapped between the surfaces. As the confined bilayers were further compressed  
37  
38 (Figure 7A Curve ②), an LPS-Ra bilayer was squeezed out with a step size  $\delta D \sim 9$   
39  
40 nm corresponding to the thickness of an LPS-Ra bilayer (as shown schematically in  
41  
42 Figure 7B), again facilitated by the enhanced acyl tail fluidity, in a process analogous  
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44 to the membrane fusion processed first observed by Horn between phospholipid  
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46 bilayers <sup>65</sup>. The second hard wall contact  $D_{0,2} = 18$  nm indicates that a single LPS-Ra  
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3 bilayer remained between the surfaces at the highest compression force at this contact  
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5 spot.  
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7  
8 The force profiles varied somewhat at different contact spots in 2.5 mM CaCl<sub>2</sub> at 40  
9 °C, with different onset surface separations for the pre-hard wall repulsion and the  
10  
11 number of steps (i.e. the number of the bilayers being squeezed out) in the fusion  
12  
13 process, suggesting some inhomogeneities in the surface coverage of the adsorbed  
14  
15 LPS-Ra vesicles, also consistent with AFM images (cf. Figure 4B,C). Meanwhile, the  
16  
17 step size in the force profiles was very similar at different contact spots, i.e.  $\delta D = 8\text{-}10$   
18  
19 nm was consistent throughout, corresponding to thickness of an LPS-Ra bilayer.  $F_N/R$   
20  
21 vs.  $D$  profile at a different contact spot (Curve ③; main figure in Figure 7 and inset). In  
22  
23 this case, the minimum absolute hard wall surface separation was  $D_0 \sim 10.0$  nm at a  
24  
25 compression force of up to  $F_N/R = 1,000$  mN m<sup>-1</sup>, indicating the firm attachment of an  
26  
27 LPS-Ra layer between the surfaces, which could not be squeezed out. Prior to the  
28  
29 ultimate hard wall, up to 4 steps in the force profiles were observed (**a – d** demarcated  
30  
31 by vertical dot-dashed lines; more distinct on the log-linear plot in the inset). The first  
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33 step (**a**) was initiated at  $F_N/R \sim 3\text{-}4$  mN m<sup>-1</sup> at  $D = 46.5$  nm with a step size  $\delta D = 18\text{-}$   
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43 19 nm, which could



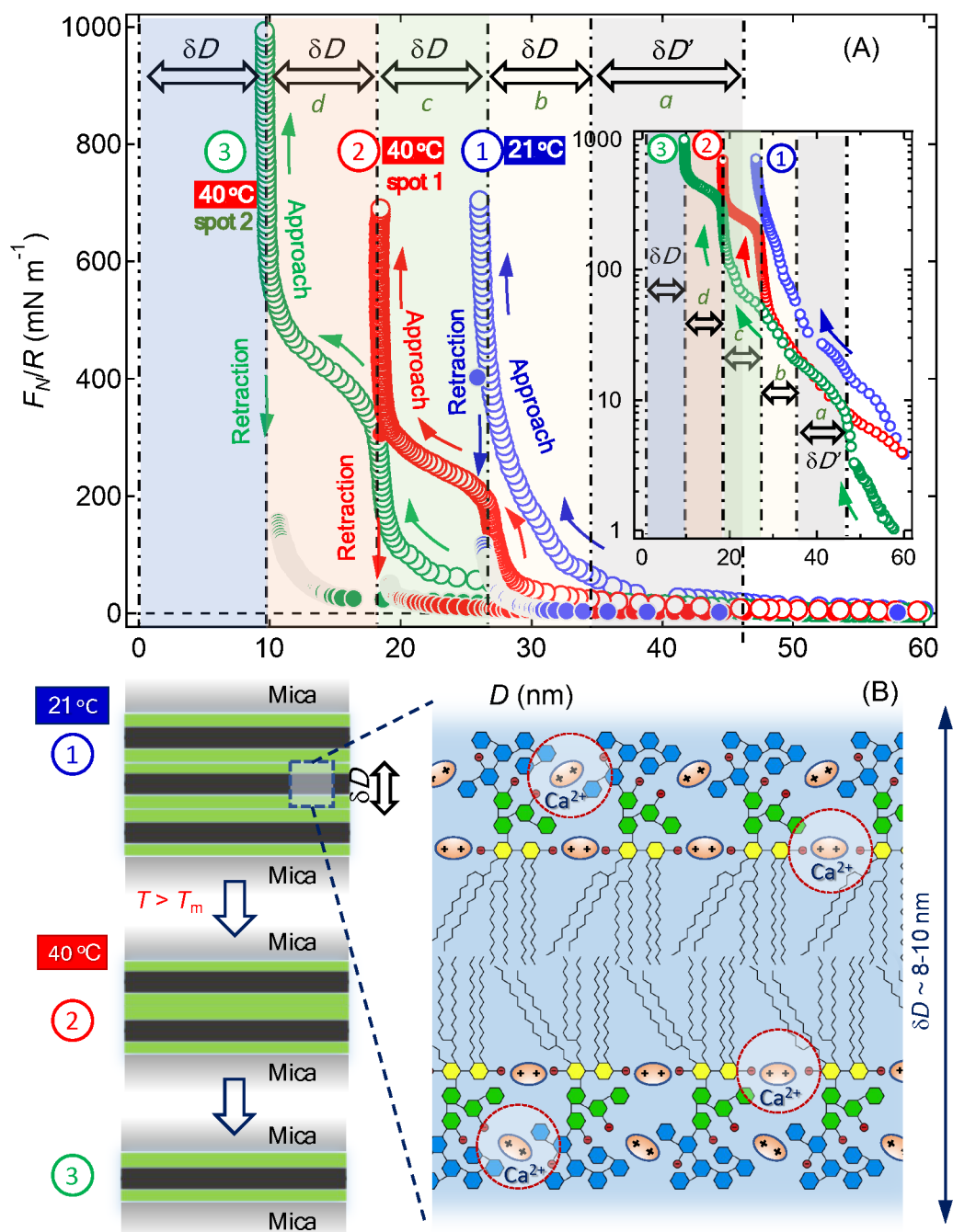


Figure 7. (A) Comparison of the normal surface force normalised by the contact radius vs. surface separation ( $F_N$  vs.  $D$ ) between mica immersed in  $0.1 \text{ mg mL}^{-1}$  LPS-Ra dispersions in  $2.5 \text{ mM CaCl}_2$  at  $21^\circ\text{C}$  (Curve ①) and  $40^\circ\text{C}$  (at two different contact spots; Curve ② and ③) upon approach (empty circles) and retraction (filled circles) of the surfaces. The inset shows the corresponding log-linear plot for the approach force profiles. As demarcated by the vertical dot-dashed lines, the discrete steps of step size  $\delta D$  or  $\delta D'$  are evident in the force profiles at  $40^\circ\text{C}$  ( $>$  melting temperature  $T_m = 36^\circ\text{C}$  of the acyl chain of LPS-Ra). Labels a-b indicate the four steps in Curve ③. (B) Schematic showing 3 LPS-Ra



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3 bilayers being trapped between two mica surfaces (shaded grey rectangles as labelled) at 21 °C, and  
4 the hard wall contact at 40 °C after bilayers of LPS-Ra are squeezed out due to the enhanced fluidity  
5 of LPS-Ra acyl tails. The schematic on the righthand side of (B) shows an LPS-Ra bilayer with a thickness  
6 of  $\delta D = 8\text{-}10$  nm, with the bridging role of  $\text{Ca}^{2+}$  between phosphate groups in the core and lipid A  
7 regions also highlighted.  
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14 be due to compression or removal of a loosely attached top layer or vesicle fragments.  
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16 This was followed by two not-as-well-defined steps (**b** and **c**) onsetting at  $D = 28$  nm  
17 and  $D = 18.5$  nm, respectively, with a step size of  $\delta D = 9.5$  nm, before a well-defined  
18 step (**d**) was initiated at  $F_N/R \sim 350$  mN m<sup>-1</sup> between  $D = 18.5$  nm and  $D = 10.5$  nm ( $\delta D$   
19 = 8.0 nm). This is consistent with progressive squeeze-out of LPS-Ra bilayers before  
20 the final more complete bilayer was squeezed out in the fusion process.  
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29 Rinsing the system with water did not alter the force profiles significantly, further  
30 underlining the high stability of the LPS-Ra surface layer in the presence of  $\text{Ca}^{2+}$ .  
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33 Interestingly, the effect of temperature on the behaviour of the LPS-Ra surface layer  
34 was largely reversible, when several heating-cooling cycles were carried out  
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39 successively.  
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42 Our SFA results indicate the presence of 3-4 stacked LPS-Ra layers of thickness  $\delta D$   
43 = 8-10 nm between the surfaces, with possible further loosely bound layers of LPS-  
44 Ra aggregate fragments atop. Both the minimum hard wall surface separation  $D_0$  and  
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49  $\delta D$  correlate well with the thickness of a LPS-Ra bilayer as determined from using  
50 crystallography data (i.e. the length of a single LPS-Ra molecule  $\sim 4.4$  nm)<sup>66</sup>.  
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54 Temperature dependent fusion of supported 1,2-dimyristoyl-*sn*-glycero-3-  
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3 phosphocholine (DMPC) bilayers has been previously observed with SFA – i.e. in the  
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5 fluid state but not in the gel state, attributed to increased chain fluidity with the  
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7 temperature both the tail melting point<sup>67</sup>. Layers of approximately 9 nm of LPS-Ra on  
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9 mica have been reported by Tong et al.<sup>31</sup> using AFM to study the adsorption behaviour  
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11 of LPS-Ra liposomes on mica in a buffer (25 mM KCl, 5 mM HEPES, pH 7.4). They  
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13 attributed the formation of the 9 nm LPS-Ra surface layer to the rupture and spreading  
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15 of the liposomes on the surface. In addition, AFM imaging also revealed areas of the  
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17 surface covered with double-bilayers. Such bi-lamellar LPS-Ra aggregates of 8-9 nm  
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19 in thickness have also been visualised using cryo-electron microscopy<sup>56</sup>. The  
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21 observed structural transformation at 40 °C is also relevant to understanding the  
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23 functionality of bacterial outer membranes and their interactions with antimicrobial  
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25 agents. For instance, Parachini et al.<sup>68</sup> observed that thermally induced gel-to-liquid  
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27 crystalline phase transition in the asymmetric LPS-DPPC bilayers affected the  
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29 penetration by Polymyxin B, an antibiotic used for resistant Gram-negative infections.  
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32 Lu et al.<sup>27</sup> and Tong et al.<sup>31</sup> found an LPS-Ra surface layer on mica even in the  
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34 absence of calcium cations. The very high ionic strength in the HEPE buffer used by  
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36 Lu et al.<sup>27</sup> might have sufficiently suppressed the repulsion between LPS-Ra and mica.  
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38 Tong et al.<sup>31</sup> used “a force as small as possible” to obtain their images, and indeed,  
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40 they demonstrated that it was possible to squeeze out the LPS-Ra layer by applying  
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42 a higher force. Our SFA results indicate the formation of a surface layer even in water,  
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44 which was however weakly adsorbed to mica, readily squeezed out. In contrast, our  
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46 SFA and AFM imaging results indicate that Ca<sup>2+</sup> significantly enhanced the adsorption  
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3 of LPS-Ra vesicles and also the binding of the LPS-Ra bilayer to mica, which was  
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5 retained even at the highest load applied. This is due to the bridging effect mediated  
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7 by  $\text{Ca}^{2+}$  between the anionic groups in the LPS core and lipid A, and between the LPS  
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9 core and the negatively charge mica<sup>30</sup>. Furthermore, a significant increase in the zeta  
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11 potential of LPS-Ra in solution from -56.7 mV in pure water to -16.4 mV in 2.5 mM  
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13  $\text{CaCl}_2$  solution at RT points to the reduced electrostatic repulsion between LPS-Ra  
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15 and the negatively charged mica substrate.  
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## 23 **Concluding remarks**

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26 LPS-Ra, a mutant bacterial lipopolysaccharide comprising lipid A and inner and outer  
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28 cores (i.e. without the O-antigen group), represents a model molecule to study self-  
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30 assembly properties of bacterial LPS in solution and at interfaces. These properties  
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32 are critically important to understanding the septic effects of LPS and the structural  
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34 integrity of Gram-negative bacterial membranes in which LPS is a major structural and  
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36 functional component – which can be targeted in novel antimicrobial strategies. Here,  
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38 the interactions between the surface layers of LPS-Ra on mica have been studied  
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40 using a surface force apparatus (SFA) in pure water and 2.5 mM  $\text{CaCl}_2$  (close to the  
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42 physiological  $\text{Ca}^{2+}$  concentration), at RT (21 °C) and at 40 °C (above the melting  
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44 temperature of the LPS-Ra acyl chains). Complementary dynamic light scattering  
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46 (DLS) measurements indicated that LPS-Ra aggregates formed at RT, with the  
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48 aggregate hydrodynamic diameter increasing from  $d_h \sim 28$  in pure water to  $d_h \sim 80$  nm  
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50 as the  $\text{CaCl}_2$  concentration was increased to 10 mM. The corresponding zeta potential  
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3  $\zeta$  data indicated binding of  $\text{Ca}^{2+}$  to LPS-Ra, causing  $\zeta$  value to become less negative,  
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5 changing from  $\sim -85$  mV to  $\sim -15$  mV. AFM imaging revealed little adsorption of LPS-  
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7 Ra aggregates on mica in pure water, but significant adsorption of a layer of  
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9 spherical/ellipsoidal aggregates on mica in  $\text{CaCl}_2$ . The length of an LPS-Ra molecule  
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11 is 4.4 nm; we thus suggest that vesicles/liposomes of LPS-Ra were formed in aqueous  
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13 media.  
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18 The corresponding SFA force profiles were consistent with the adsorption behaviour  
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20 of the LPS-Ra as observed from AFM imaging. That is, at 21 °C the LPS-Ra  
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22 aggregates were readily squeezed out between surfaces in pure water, whereas a  
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24 hard wall repulsion at surface separation  $D_0 = 30\text{-}40$  nm in 2.5 mM  $\text{Ca}^{2+}$  indicate the  
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26 strong LPS adsorption of either a monolayer of flattened LPS-Ra aggregates on each  
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28 surface or  $\sim 4$  LPS-Ra bilayers trapped between the surfaces. The role of  $\text{Ca}^{2+}$  of  
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30 surface structuring of LPS-Ra vesicles is significant and twofold. First,  $\text{Ca}^{2+}$  bridges  
31  
32 the negatively charged phosphate groups between LPS-Ra molecules, which  
33  
34 stabilises the LPS-Ra vesicles against rupture at the surface. Secondly,  $\text{Ca}^{2+}$  similarly  
35  
36 bridges between negatively charged LPS-Ra outer core and negatively charged mica,  
37  
38 facilitating the strong adsorption of the LPS-Ra aggregates, also observed with AFM.  
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40 Considering that many biological surfaces are slightly negatively charged, this  
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42 observation has implications to the mechanism of LPS sepsis.  
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51 At 40 °C ( $T > T_m$ ), the fluidity of the LPS-Ra acyl tails was greatly enhanced,  
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53 manifesting in multilayer steps in the surface force profiles as the surface aggregate  
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55 layers were compressed, due to LPS-Ra bilayers being squeezed out in a process  
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3 similar to membrane fusion. Such layering behaviours also confirm bilayers as a  
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5 structural feature in LPS-Ra aggregates, thus substantiating our suggestion that LPS-  
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7 Ra vesicles formed in aqueous media, consistent with the DLS hydrodynamic diameter  
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9  $d_h$  data. These unprecedented SFA results provide insights on the stability and  
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11 structural integrity of LPS-Ra membranes – how it could be challenged and tuned by  
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13 the presence of  $Ca^{2+}$  and modest temperature changes. Although the chain-melting  
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15 effect has been induced by temperature elevation in this study, one could envisage  
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17 alternative routes to impart similar effects, e.g. by inserting nano-objects of certain size  
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19 and surface chemistry into the membrane to induce structural disorders<sup>69-72</sup>.  
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26 LPS-Ra shares many structural and chemical features with other rough LPS mutants  
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28 and smooth bacterial LPS. The well-defined differences between these mutants would  
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30 provide a framework in which the roles of different structural features (e.g. the inner  
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32 core, outer core, and O-antigen) can be studied systematically , and the effect of  
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34 solution conditions (such as the temperature, ionic strength, pH, and the presence of  
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36 nanoparticles and antimicrobial proteins) on the structure of the LPS membranes can  
37  
38 be studied using quantitative and rigorous physicochemical techniques, such as the  
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40 Israelachvili surface force apparatus (SFA).  
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## 35 **Dedication**

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39 We dedicate this paper to the memory of Jacob Israelachvili, a pioneering and inspiring  
40 scientist in the measurement and understanding of surface forces.  
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