Phospholipid membranes as substrates for polymer adsorption

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A largely unsolved problem in soft materials is how surface reconstruction competes with the rate of adsorption. Here, supported phospholipid bilayers of DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) were employed as substrates for the adsorption of a weak polyelectrolyte, polymethacrylic acid, whose time-dependent ratio of charged to uncharged functional groups served to probe the local dielectric environment. Chains that encountered sparsely covered surfaces spread to maximize the number of segment–surface contacts at rates independent of the molar mass (which was varied by a factor of 30), but dependent on the phase of the lipid bilayer, gel or liquid crystal. Surface reconstruction rather than molar mass of the adsorbing molecules seemed to determine the rate of spreading. The significance of these findings is the stark contrast with well-known views of polymer adsorption onto surfaces having structures that are 'frozen' and unresponsive, and is relevant not just from biological and biophysical standpoints, but also in the formulation of many cosmetics and pharmaceutical products.

Polymers are known to adsorb prodigiously from solution, because a small adsorption energy per segment adds up to a large adsorption energy per molecule[1,2]. In the field of polymer science, this is traditionally considered to occur on surfaces having a 'frozen', unresponsive structure, and definitive treatises exist on this subject[1,2]. In contrast, in the study of phospholipid membranes, drug delivery and gene therapy, interactions with polymers are known phenomenologically to have the capacity to make membranes leaky, for example, for the outflow of drugs from vesicles[3] or the inflow of encapsulated DNA into cells[4]. In these cases the membrane structure is clearly disrupted. Bacteriocidal action has even been demonstrated[5] by polymer disruption of phospholipid membranes.

It is interesting that these different communities have developed with little cross-talk. The polymer science and drug-delivery communities have focused on extreme limits—the polymer science community focusing on the polymer side of the interface, the biologically minded community focusing on the practical consequences when membranes are disrupted. Here, we are concerned with the 'middle ground'—a membrane surface that responds to the adsorbate but without being penetrated or destroyed. In the field of surface science, the dynamics of adsorption and surface equilibration when the surface possesses reciprocal mobility is also a largely unsolved problem[6]. In this study of surface equilibration dynamics at a responsive surface, we find patterns of physical behaviour that differ remarkably from what is characteristic of adsorption on to frozen surfaces[1,2,7–9].

Single phospholipid bilayers supported on flat, solid substrates[10,11] offer model systems for studying responsive surfaces. In favourable systems they can be switched from the 'high-temperature' liquid crystalline (LC) to the 'low-temperature' gel phase within the range of room temperature without changing the chemical composition. In addition, the fluidity of the phospholipid bilayers supported on a solid surface is similar to that of freestanding liposomes due to lubrication by a nanometre-thin water layer between the bilayers and the hard solid substrate, but the supported bilayers have the advantage over liposomes of having a well-defined geometry. A limited amount is known at the molecular level regarding the response of bilayers to adsorption. Membrane leakiness can be modified[3–5], the size and geometry of liposomes can change on the adsorption of polymer[12,13], and membrane stiffness can change[14], but these properties are macroscopic. Here, taking advantage of the chemical information afforded by the method of Fourier

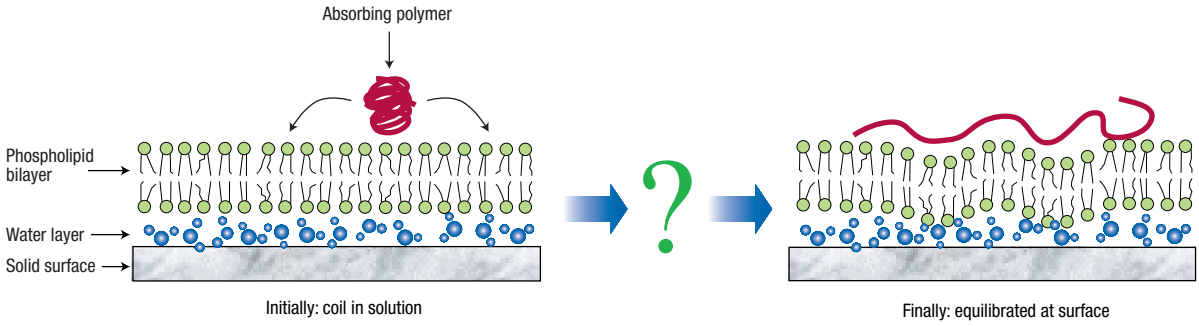

Figure 1 Schematic diagram of the adsorption and subsequent conformational equilibration of flexible polymers at a supported phospholipid membrane. The initially bare membrane was exposed to dilute polymer solution. The amount of polymer adsorbed and the dynamics of equilibration after adsorption was studied. In most of the experiments, the surface was 'starved' of polymer to produce sparse coverage, <5% of a monolayer, in order to study the 'spreading' of adsorbed chains towards the anticipated pancake configuration.

transform infrared spectroscopy in the mode of attenuated total reflection (FTIR–ATR), we study membrane reorganization as shown by local properties. We specifically look at the infrared dichroism of the phospholipid headgroup, and the degree of acid dissociation when a weak polyelectrolyte adsorbs. To our knowledge, no previous experimental study has concerned the kinetics of surface reorganization. Our experimental findings complement the growing number of theoretical studies concerning membranes decorated with anchored or adsorbed polymers[15–21], from which the prediction emerges that adsorption modifies the local bending rigidity and the local spontaneous radius of curvature of the membranes.

The model system was selected on the basis of the following considerations. First, for simplicity, the membrane should be comprised of a single lipid, not a mixture. Second, the complexity of having a net electrical charge should be avoided. (We have preliminary evidence of a strong influence of electrostatic interactions with cationic phospholipids, but the parameters of the contrast are not simple.) Third, the lipid should present a phase transition at an experimentally convenient temperature so that the influence of lipid mobility could be studied. Finally, the experimental method should be capable of following both the mass adsorbed and the kinetics of spreading and surface reconstruction. This was achieved by *in situ* measurements using infrared spectroscopy.

With these considerations in mind, supported bilayers were prepared of the phospholipid DMPC (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), which has a fluid–solid phase transition at the experimentally convenient temperature of 23 °C. Figure 1 illustrates schematically the process under study. A supported lipid bilayer was exposed to dilute polymer chains in solution, resulting in adsorption, and the dynamics of the passage from the initial to the final state were studied. Figure 2 illustrates the unanticipated result that the adsorption was 'high affinity' (that is, a large amount of PMA adsorbed even at the earliest measurement times, although the solution concentration was only 10 p.p.m. and 1,000 p.p.m.), and that the overall amounts adsorbed were similar to those at frozen surfaces[1,2] when the solution concentration is similar. What was the driving force for adsorption? The headgroups of the DMPC forming the bilayer were dipolar, being positive on the termini ($-N(CH_3)_3^+$ units) and negative a few angstroms underneath ($-PO_2^-$ units). Although a contribution from hydrophobic attraction cannot be excluded, the main driving force was probably electrostatic attraction between the negative charges on the polymer chains and the dipoles in the headgroups of the lipid bilayer.

In the field of polymer science, it is known that the acidic groups on a weak polyelectrolyte dissociate to an extent that responds to the local environment; for example, dissociation of carboxylic acid groups can vary between minimal and near-complete[22,23]. Also from Fig. 2, it can be seen that the intensity ratio of the charged to uncharged adsorbed carboxylic groups underwent an overshoot as adsorption proceeded uninterruptedly (in this example, the bilayer was in the gel phase). However, the overshoot was less pronounced the higher the solution concentration. The more rapid adsorption at higher concentrations suggested that the overshoot reflected two competing populations of chains, some that deposited when the surface was sparsely occupied, and others that deposited when the coverage was high.

To test this hypothesis, the surfaces were 'starved' of polymer by depositing an amount too small to saturate them. Figure 3 shows an experiment in which, after PMA was allowed to adsorb to just 5% of the saturated amount, the solution of polymer was replaced by pure buffer solution. Thereafter the total mass adsorbed was constant (Fig. 3a), but the intensity ratio of the charged and uncharged carboxylic groups adsorbed continued to decrease (Fig. 3b). In this situation, where surface coverage was limited to much less than a monolayer, one expects the

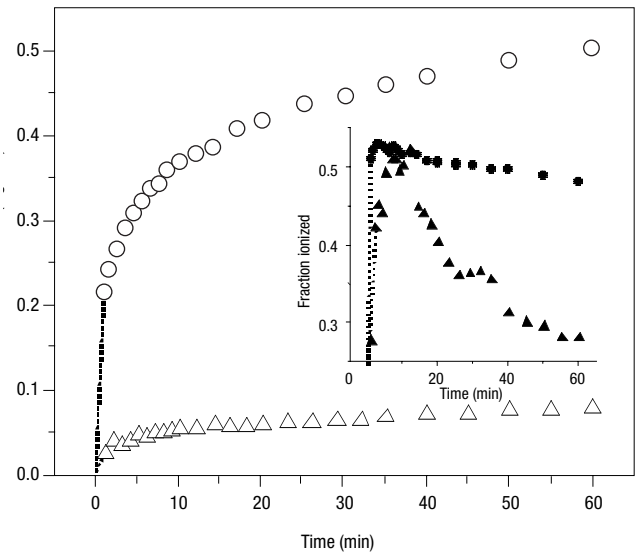

Figure 2 Mass of polymethacrylic acid (PMA) adsorbed on to the supported lipid bilayer surfaces versus time. The inset shows the ionization of the carbonyl group versus time. PMA solutions with two different concentrations, 1 mg ml^{-1} (circles) and 0.01 mg ml^{-1} (triangles) were used, buffered by 150 mM phosphate salt at pH = 6.0, temperature 15 °C.

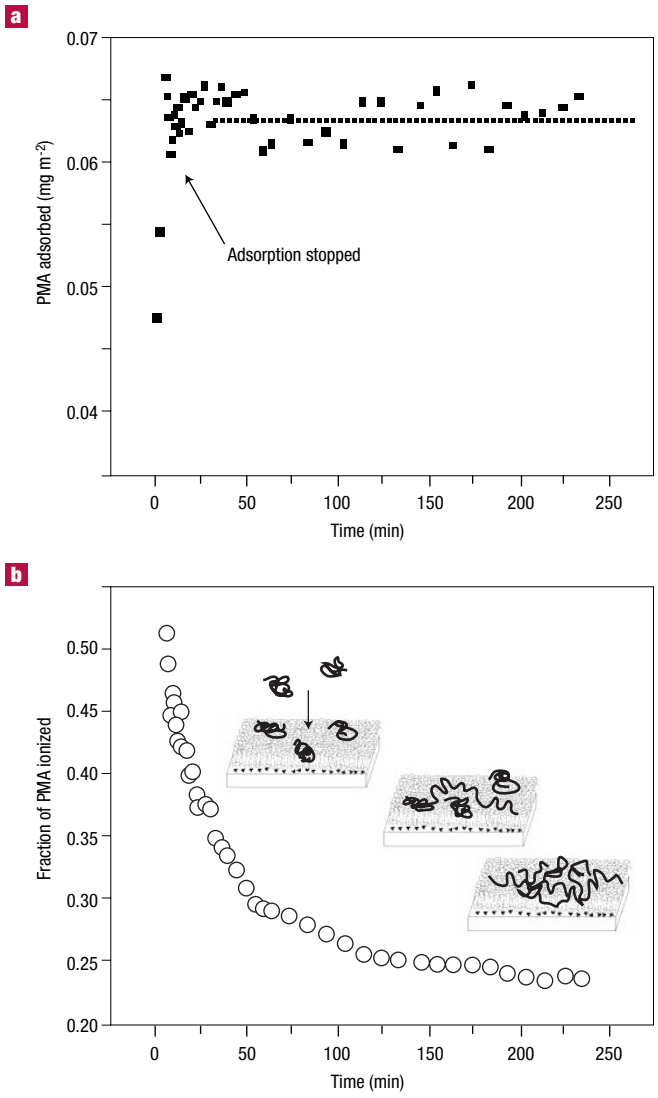

Figure 3 Ionization of the carbonyl group plotted against elapsed time for a surface starved of PMA. The weight-average molecular weight M_w was 40,000 g mol^{-1}, and the polydispersity M_w/M_n was 1.02 (where M_n is the number-average molecular weight). The polymer was allowed to adsorb, from 0.1 mg ml^{-1} in aqueous D_2O solution buffered at pH = 6.0, on to a supported lipid bilayer of DMPC at 15 °C such that the bilayer was in the gel phase. **a**, The time evolution of the mass of adsorbed PMA. Adsorption was allowed to proceed for 4 min, to ~5% of equilibrated total surface coverage, at which point the solution was replaced with the same buffered solution but without polymer. The dotted line indicates that total mass adsorbed remained constant. **b**, Fractional ionization of PMA plotted against time elapsed. The ionization decreased over a period of more than 2 h. The ionization in bulk solution was 0.58. A schematic series of pictures of the hypothetical spreading process is included.

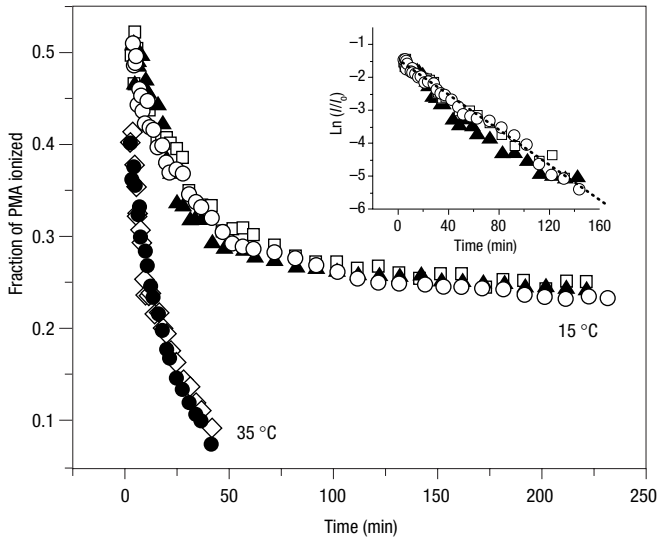

Figure 4 Ionization of the carbonyl group plotted against elapsed time for varying molecular weights of PMA spread on to starved bilayer surfaces. In experiments with DMPC in the gel phase at 15 °C, the M_w of PMA used was 13,000 g mol^{-1} (filled triangles), 40,000 g mol^{-1} (open circles) and 327,000 g mol^{-1} (open squares). With DMPC in the LC phase at 35 °C, the M_w of PMA used was 40,000 g mol^{-1} (filled circles) and 327,000 g mol^{-1} (open diamonds). The adsorption conditions were the same as described in Fig. 3. The inset shows the normalized ionization I/I_0, the ratio of the actual fractional ionization to the ionization in bulk solution (0.58), plotted semilogarithmically as the function of elapsed time, for those cases when PMA adsorbed onto the bilayer in the gel phase. Curves representing different molecular weights fell on to one (dotted) line. The time constants were $\tau_s = 15 \pm 3$ min and 30 ± 2 min in the LC and gel phase, respectively.

polymer to adopt a flat 'pancake' conformation at equilibrium[1]. The equilibration process shown in Fig. 3, in the direction of additional segment–surface contacts, was slower than the rate of additional mass deposited when the supply of polymer was uninterrupted (Fig. 2). This confirmed the hypothesis that the findings obtained during uninterrupted adsorption reflected the mismatch between the rates of spreading and additional deposition. For the data in Fig. 3, we suppose that the spreading of adsorbed chains, that is, the increase of segment–surface contacts from the dipolar headgroups, produced the observed changing ratio of charged to uncharged ionized groups. The puzzle is to understand why the rate of spreading was so slow, because local diffusion times of lipids within DMPC bilayers are far more rapid than this[24,25].

Let us take stock. An alternative interpretation might be that the chains of PMA penetrated into the hydrophobic core of the bilayer such that the observed reduced ionization reflected the lower local dielectric constant. To test this possibility, parallel control experiments were performed using a more hydrophilic polymer, polyacrylic acid. Similar results were obtained, hence eliminating this line of argument. A second alternative interpretation might be that the polymer chains burrowed beneath the lipid bilayer to bind with the underlying Si or Ge. To test this possibility, experiments were performed using a polymer cushion of polystyrenesulfonate (PSS) before depositing the phospholipid bilayer. The charge of both PMA and PSS is negative, which would prevent association. The similar results obtained from this control experiment appeared to eliminate that line of argument also. Instead, it appears that the polymer segments associated with the outermost headgroup region of the lipid bilayers. Finally, one may wonder if adsorption induced changes of the average area per phospholipid. But the infrared measurements in the C-H vibration region (the lipid tails) showed no shift in peak position or intensity change during the polymer adsorption process, which argues against packing density changes of the lipid bilayer.

Theory[1] and computer simulations[9] predict that the spreading time, τ_s, when a polymer molecule spreads on to a solid surface, slows with increasing degree of polymerization N. For example, in a reptation-based theory[1], it scales as N^2x^{-5}, where x denotes the level of surface unsaturation. In our case, the dependence on x can be eliminated, because the surface coverage was maintained far below saturation. We proceeded

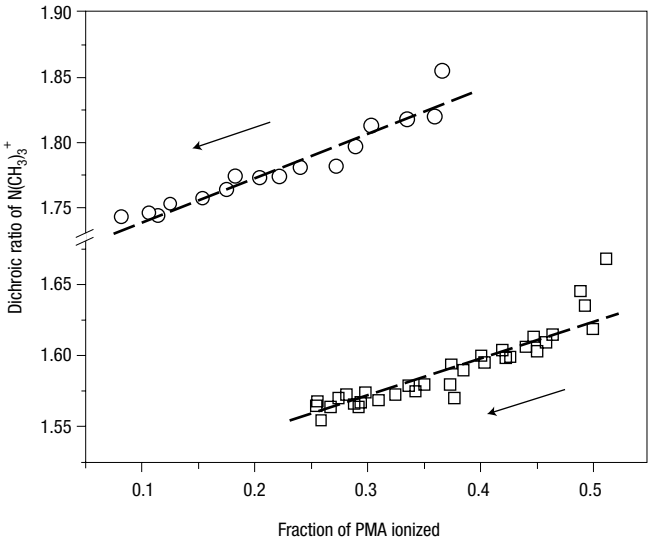

Figure 5 Dichroic ratio of the $-N(CH_3)_3^+$ asymmetric stretch vibration at 970 cm^{-1} of DMPC coated with adsorbed PMA chains in the LC phase (circles) and gel phase (squares), plotted against the fractional ionization of adsorbed PMA during spreading onto starved surfaces after the same adsorption procedure described in Fig. 3. Dashed lines show the linear relationship between dichroic ratio and fractional ionization. The PMA had M_w of 40,000 g mol^{-1}. The adsorption condition was 0.1 mg ml^{-1} in aqueous D_2O solution buffered at pH = 6.0.

to vary N, as summarized in Fig. 4. First, in the gel phase of the lipid bilayers (15 °C), we varied N by a factor of 30 (increasing the molecular weight from 13,000 to 327,000 g mol^{-1}, N = 120 to 3,030) but without affecting the rate of spreading. To test this conclusion, temperature was raised to place the lipid bilayers in the LC phase (35 °C). Spreading was indeed more rapid, but again without dependence on N. The inset of Fig. 4, showing a semilogarithmic plot of the data for spreading in the gel phase, illustrates that these processes were exponential. One might indeed wonder whether N ever mattered, so to further investigate whether N has any effect, experiments were performed with a monomer analogue of the polymer, acetic acid (N = 1), at solution concentrations up to 6% (1 M), but the monomer analogue failed to adsorb, as expected because the sticking energy per segment is weak when a polymer adsorbs. Adsorption results because the weak sticking energy of individual segments along the chain adds up to a substantial sticking energy per molecule. The lack of molecular weight dependence when the chains were long enough for adsorption, and the simple exponential dependence on elapsed time, contrast in both respects with behaviour when polymers adsorb at frozen interfaces[1,7–9].

But this is not so if the underlying material is responsive. In essence, the lack of dependence on molecular weight is reminiscent of the N-independent spreading rate recently discovered when liquid droplets spread on a viscoelastic surface[26,27]. The usual strong viscosity dependence of the spreading rate, for cases where the surface is a hard solid, is lost. The rate-limiting step is instead observed to be a viscoelastic response of the underlying material[26,27].

In a lipid bilayer, what is the analogue of surface viscoelasticity? How does a membrane surface respond to the presence of polymer chains? Our infrared measurements could also detect nitrogen–carbon vibrations characteristic of the $N(CH_3)_3^+$ terminus of the lipid headgroups, which had direct contact with the chains, so that measurements using polarized radiation could be used to measure their mean orientation with respect to the surface. For this purpose, the dichroic ratio D was defined as the ratio of infrared absorption to radiation polarized in perpendicular and parallel directions. In Fig. 5, D is plotted against the ionization of the polymer during the adsorption process (note that the measurements of D are more scattered than those of ionization because the infrared absorption peaks were weaker). With small deviations at the earliest times, ionization changes were in one-to-one linear correspondence with dichroism changes. Note that the relative change of D was small because coverage by polymer was sparse. The mean tilt angle to the surface normal, in the gel and LC phases, respectively, was 46.6° and 43.7° for the bare lipid (D = 1.71 and 1.89). It was 56.9° and 50.4°, respectively, at saturated surface coverage (D = 1.29 and 1.52). This is evidence of direct coupling between adsorption and surface reconstruction. It constitutes further evidence, given the lack of N-dependence, that the surface reconstruction process was rate-limiting.

Furthermore, because the intensity of infrared absorption was calibrated to the mass of lipid and polymer involved, the observed changes in D and ionization enable the estimation of the stoichiometry of interaction between polymer and lipid headgroups assuming linear additivity. The final states imply that five lipid headgroups were influenced, on average, by each charged unit of the polymer. The effect extended beyond the direct charge–charge coupling between a single polymer segment and single lipid headgroup. Extrapolating from the theoretical prediction that polymer adsorption stiffens a membrane locally[15–21], we conjecture that the surface reorganization process involved changes of local curvature and local stiffness, during the time that the polymer chains unwound from the three-dimensional solution conformation towards the two-dimensional pancake conformation. The data in Fig. 4 show that, during this process, segments of the chains bound to an increasing number of lipid headgroups.

Looking to the future, these measurements have a bearing on understanding the binding of polymers to lipid membranes, which is a subject of interest not just from biological and biophysical standpoints[28] but also for formulating many cosmetics and pharmaceutical products[29]. We expect by the same logic that surface reorganization should compete with adsorption when DNA and proteins adsorb to lipid membranes. The existing experiments have followed the equilibrated situations only. Unfortunately we have at present no *in situ* experimental method capable of following such kinetics because in those situations the charge on the adsorbate is permanent. A distinguishing (and unique) feature of this study is that the spreading process could be studied because electrical charge from ionization responded to the local dielectric environment.

The systematic, regular, quantitative patterns of behaviour reported here signifies that, although adsorption on these responsive surfaces differs strongly from polymer adsorption on traditional 'hard' surfaces, the 'middle ground' between extreme cases discussed in the introduction surely exists. The spatial self-organization of adsorbates on fluid bilayers induced by local curvature has been considered theoretically[16,17]. Tentatively, we conjecture that this may be the reason for the long spreading times that we observe, which exceed the characteristic diffusion times of lipids within a membrane.

METHODS

PREPARATION OF THE BILAYERS

Small unilamellar vesicles of DMPC (Avanti Polar Lipids, Alabaster, USA) were prepared by the extrusion method[30], then injected into a thermostatted cell[8] containing a Ge or Si crystal. Spontaneous spreading of these vesicles produced a single bilayer supported on the crystal. To encourage equilibration, the cell was incubated at 35 °C for at least 2 h. The excess non-fused vesicles were then rinsed out by copiously flushing the cell with phosphate buffer solution. The synthetic flexible polymers, polymethacrylic acid (PMA, Polymer Standards Service, Mainz, Germany) or polyacrylic acid (Polymer Source, Québec, Canada) were allowed to adsorb.

INFRARED SPECTROSCOPY

The FTIR spectra were collected using a Biorad FTS-60 instrument equipped with a mercury–cadmium–telluride detector. In each experiment, before the introduction of the buffered polymer solution, the spectrum of the pure lipid bilayer was collected in the presence of the same buffer solution. By switching a wire-grid polarizer (Graesby/Specac), the infrared spectra in both *p*- and *s*-polarization modes were obtained. Most experiments were performed in D_2O rather than in H_2O because D_2O was

more transparent in the frequencies of interest. The inorganic salts Na_2HPO_4 and $NaH_2PO_4 \cdot H_2O$ were used to control pH and ionic strength.

The fidelity of the single supported lipid bilayers was examined; in all experiments, the infrared intensity was consistent within ± 5%. Calibration, using methods described previously[22], showed this to be 4.1 mg m^{-2}, or 55 $Å^2$ per phosphocholine headgroup for the LC phase. In addition, when the polymer was introduced, the amount adsorbed and the degree of dissociation of the carboxylic acid group (ratio of carboxylate to carboxylic acid functional groups) were evaluated using methods described previously[22]. In the infrared spectra, no changes of peak position or peak width were observed in the C-H vibration region by polymer adsorption, suggesting no major polymer-induced changes occurred in the packing of the lipids.

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Competing financial interests

The authors declare that they have no competing financial interests.