ARTICLE

Electrostatic Interactions Are Not Sufficient to Account for Chitosan Bioactivity

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ABSTRACT Recent studies involving chitosan interacting with phospholipid monolayers that mimic cell membranes have brought molecular-level evidence for some of the physiological actions of chitosan, as in removing a protein from the membrane. This interaction has been proven to be primarily of electrostatic origin because of the positive charge of chitosan in low pH solutions, but indirect evidence has also appeared of the presence of hydrophobic interactions. In this study, we provide definitive proof that model membranes are not affected merely by the charges in the amine groups of chitosan. Such a proof was obtained by comparing surface pressure and surface potential isotherms of dipalmitoyl phosphatidyl choline (DPPC) and dipalmitoyl phosphatidyl glycerol (DPPG) monolayers incorporating either chitosan or poly(allylamine hydrochloride) (PAH). As the latter is also positively charged and with the same charged functional group as chitosan, similar effects should be observed in case the electrical charge was the only relevant parameter. Instead, we observed a large expansion in the surface pressure isotherms upon interaction with chitosan, whereas PAH had much smaller effects. Of particular relevance for biological implications, chitosan considerably reduced the monolayer elasticity, whereas PAH had almost no effect. It is clear therefore that chitosan action depends strongly either on its functional uncharged groups and/or on its specific conformation in solution.

KEYWORDS: chitosan • membrane models • Langmuir monolayers • electrostatic interactions • polyelectrolytes • bioactivity

INTRODUCTION

Chitosan is a cationic biopolymer with distinguished applications in many fields, such as antimicrobial agent (1, 2), in drug delivery (3), for transfection (4), in lowering cholesterol and fat , and tissue engineering (6). In most of these applications, chitosan has an intimate contact with cells and more specifically with cell membranes. It is thought that the mechanisms involved depend on the interactions that take place at the molecular level when chitosan approaches and lies adsorbed at the cell membrane. Because experimental techniques involving cell cultures are not able, up to now, to elucidate interactions at the molecular level, it is common to resort to cell membrane models. Cell membranes are constituted basically by a lipidic bilayer, thus thin films of phospholipids (7–9) or vesicles (10) are suitable to mimic biomembranes.

The effects from chitosan on cell membrane models have been studied using Langmuir monolayers (11–15) and uni- or multilamellar vesicles (16–19). It has been established that: (i) chitosan induces an expansion in phospholipid monolayers, which increases with chitosan concentration in the subphase up to saturation; and (ii) for condensed films, the change in the surface pressure isotherms is almost negligible (12−14). Even though the second feature pointed to the expulsion of chitosan from the interface at high level of packing, using Langmuir−Blodgett (LB) films we could conclude that chitosan stays entrapped in the phospholipid structure, being located at the subsurface of the monolayer, in contact with the phospholipid head groups. It was also inferred that chitosan can interact with membrane models in various ways, including through hydrogen bonding, van der Waals interactions, and electrostatic interactions (14, 15).

An interesting feature of chitosan action over membrane models is a considerably larger expansion for negatively charged phospholipids, owing to the chitosan cationic nature (14, 15). Moreover, specific activities such as protein sequestering from a lipidic membrane by chitosan depend on the net charge of the phospholipid headgroup (20). This highlights the major role of electrostatic interactions on the effects of chitosan on model membranes suggesting that the bioactivity of chitosan should be due to its cationic nature, which has also been stated by other authors (21–23). In fact, this has been supported by the simple fact that cationic biopolymers are not so common in nature.

To test if chitosan bioactivity is solely due to electrostatic interactions, in this work we have compared its behavior with another amine-based polycation, namely poly(allylamine hydrochloride) (PAH). PAH was chosen not only because it is positively charged over a large pH range but also because it has an amine as its charged group, like chitosan. Using this polyelectrolyte, we could investigate only the effects of the charged group, eliminating the contributions from OH groups and glucopyranose rings. As observed for chitosan, induced surface activity was also

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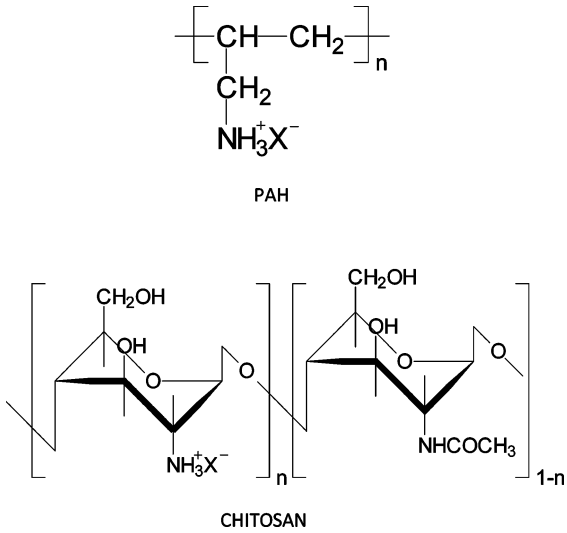

FIGURE 1. Chemical structure of the repeating units of PAH and chitosan.

observed for PAH in the presence of interfacial phospholipid films. However, the expansion and modulation of film properties, as in the in-plane elasticity, caused by this polyelectrolyte were much lower than those caused by chitosan.

EXPERIMENTAL DETAILS

The phospholipids dipalmitoyl phosphatidyl choline (DPPC) and dipalmitoyl phosphatidyl glycerol (DPPG) were purchased from Avanti, while poly(allylamine hydrochloride) (PAH) with $M_w = 15,000$ Da was acquired from Alfa Aesar, and used as received. Chitosan was obtained from Galena Química Farmacêutica (Brasil). The sample used had an acetylation degree of 22%, molecular weight 113 kDa (Mn) and polydispersity index of 4.2. The structures of PAH and chitosan repeating units are shown in Figure 1.

The Langmuir films were produced by spreading 150 μL of a 0.50 mg/mL chloroform solution of either DPPC or DPPG on the surface of a Theorell-Stenhagen buffer pH 3.0 subphase prepared using NaOH, citric acid, boric acid, phosphoric acid and ultrapure water, pH 6.0 and resistivity 18.2 MΩ cm, provided by a Millipore purification system. The pH of the buffer was adjusted to 3.0 with HCl 2M. A buffer solution with PAH or chitosan dissolved in three concentrations, namely 0.05, 0.10, and 0.30 mg/mL, were used as subphase. A Langmuir trough KSV 5000 located in a class 10 000 clean room was used in the experiments performed at room temperature, 22 ± 1 °C. The films were characterized by surface pressure and surface potential isotherms, with pressure and potential being measured with a Wilhelmy plate and a Kelvin probe, respectively. The surface compressional modulus (Cs^{1-}), also known as the in-plane elasticity, was calculated from the surface pressure isotherms using the expression: $Cs^{1-} = -A(\partial\pi/\partial A)$, where π is the surface pressure and A is the mean molecular area (24).

RESULTS AND DISCUSSION

Although the effects from chitosan on phospholipid monolayers have already been studied, a full understanding of the molecular-level interactions remains elusive. A key point is to verify whether electrostatic interactions are the major or sole responsible for the action from chitosan on model membranes. For a direct comparison we use here chitosan and PAH, which is also positively charged in an aqueous

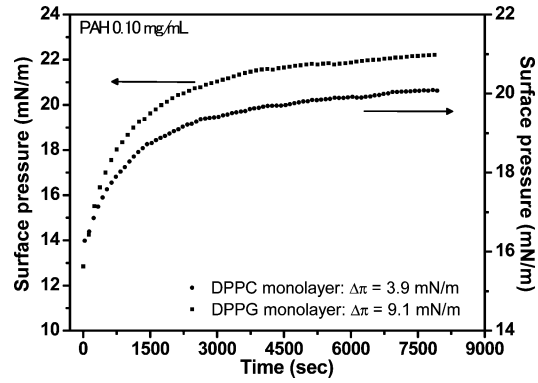

FIGURE 2. Kinetics of adsorption of PAH onto DPPC and DPPG Langmuir films. The concentration of PAH in the subphase was 0.10 mg/mL. The initial surface pressures were ca. 16 and 13 mN/m for DPPC and DPPG monolayers, respectively. The change in surface pressure caused by the polyelectrolyte adsorption ($\Delta\pi$) is given in the inset.

solution, with the charge located in the amine group, as for chitosan (Figure 1). To ensure the role of the counterion was not responsible for any possible differences between PAH and chitosan, the subphases were prepared using a Theorell-Stenhagen buffer. Both PAH and chitosan are fully protonated in the buffer, pH 3.0, as the pK_a for the amine group is 6.5 for chitosan and 8.5 for PAH.

Analogously to what occurs for chitosan, PAH on its own is not surface active and cannot form a Langmuir or a Gibbs monolayer. In subsidiary experiments, we observed that with a 0.10 mg/mL PAH concentration in the subphase, a negligible surface pressure was measured upon compressing the barriers in the Langmuir trough, even after waiting for long periods of time (results not shown). When a phospholipid monolayer is present at the air/water interface, however, PAH is adsorbed onto the film, as shown by the change in surface pressure for DPPC and DPPG in Figure 2. The total increase in surface pressure ($\Delta\pi$) for DPPG is 9.1 mN/m, to be compared with 3.9 mN/m for DPPC. This is expected because the net negative charge of DPPG headgroups favors electrostatic interactions with the positively charged amine groups of PAH. In both cases the adsorption of PAH occurs in two steps; one initial fast step followed by another slower adsorption. Around 80% of the total $\Delta\pi$ was attained within 600 s. Hence, it is safe to assume that the effects caused by PAH on the phospholipid films were all measured after most of the polymer had migrated to the surface, as the waiting time for chloroform evaporation before compression was 900 s in every further compression isotherm.

The effects of PAH on the surface pressure isotherms of DPPC and DPPG monolayers are, however, much smaller than those of chitosan, as shown in Figure 3. For the zwitterionic DPPC, Figure 3A indicates that PAH hardly caused any change in the isotherms, regardless of its concentration. Small changes are noted for DPPG in Figure 3B when the PAH concentration is 0.30 mg/mL, for which additional effects appear to exist, as observed in the surface potential isotherms to be discussed later on. Nevertheless, even these changes are much smaller than those induced by chitosan, as it is clear in Figure 3B. It is stressed that the

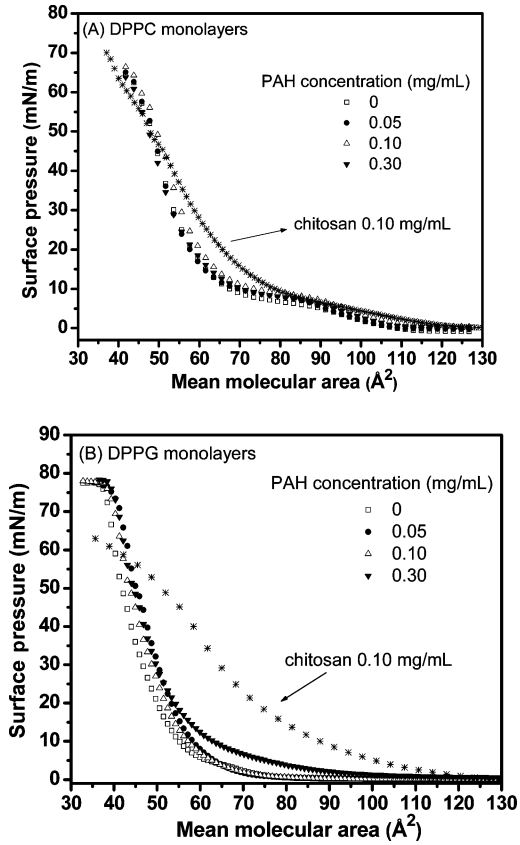

FIGURE 3. Surface pressure−area isotherms for (A) DPPC and (B) DPPG monolayers formed on Theorell buffer (pH 3.0) solution and PAH solutions on the same buffer (the concentration of PAH is indicated in the inset). For comparison, the isotherms for the Langmuir films on a chitosan-containing subphase are also shown.

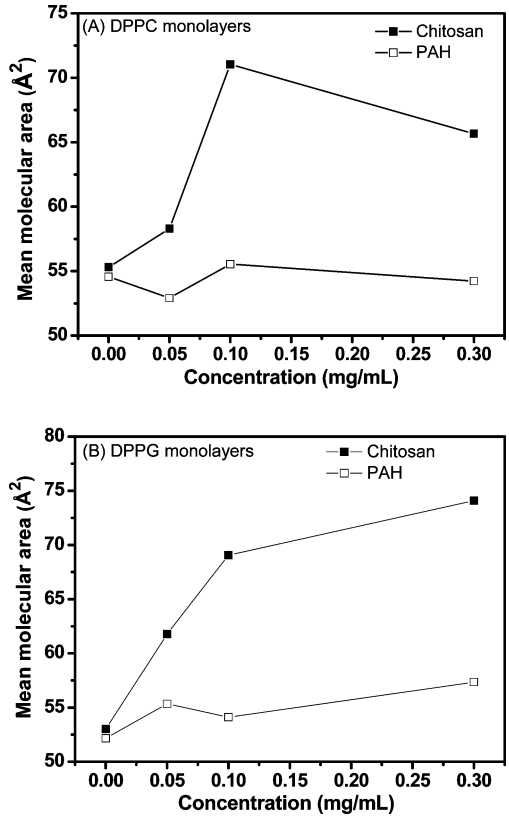

FIGURE 4. Mean molecular area per phospholipid molecule for (A) DPPC and (B) DPPG Langmuir monolayers, at 15 mN/m, as a function of concentration of the polyelectrolytes in the subphase. The results for chitosan were taken from ref 12.

isotherms in Figure 3 are reproducible, which was verified by repeating the compression−decompression cycles several times.

Aoki and co-workers have recently reported effects of PAH on DPPG Langmuir monolayers (25), in which the expansion in the surface pressure−area isotherm for a PAH concentration of 0.10 mg/mL was larger than observed here. In addition, a second phase transition around 40 mN/m appeared in the isotherms, which was attributed to the expelling of PAH from the interface at close packing. The distinct behavior for PAH in ref (25) can be ascribed to the use of a different subphase. Instead of a Theorell buffer pH 3.0 used in the present work, they employed pure water with pH 5.6. It is possible that an almost neutral pH may have induced further surface activity of PAH which is not observed in the Theorell buffer.

The differences between PAH and chitosan in Figure 3 may be better visualized by plotting the area per phospholipid molecule at a fixed pressure versus the concentration of PAH or chitosan in the subphase. This is illustrated in Figure 4 for a pressure of 15 mN/m, which confirms that the effects on DPPG are larger than on DPPC, because of the charged headgroups of DPPG. Most importantly, PAH has a much lower effect than chitosan. The surface pressure of 15 mN/m was chosen because the large effects observed at this pressure mean that strong interactions, probably with penetration into the monolayer, take place. Therefore, we wished to analyze a case of interactions close to their maximum. This behavior applies to other values of surface pressure, except for very high pressures in which the effects caused by PAH and chitosan are very small (results not shown), because PAH and chitosan are expelled from the interface, lying on the subsurface. As regards the concentrations of PAH in the subphase in this study, they were chosen to allow for a comparison with previous work with chitosan. Within the concentration range used, the phospholipids were able to induce surface activity on chitosan, which is not surface active. Above this range, the viscosity of the resulting solution is too high. Moreover, considering that the saturation concentration for chitosan is 0.20 mg/mL, working well above it would only bring disadvantages.

The much smaller changes induced by PAH−in comparison to chitosan− on the mechanical properties of phospholipid monolayers were corroborated with the analysis of the compressional modulus, also known as in-plane elasticity. Figure 5 shows that the various concentrations of PAH had little effect on the in-plane elasticity, whose maximum values were very similar to the neat monolayers and remained at the same areas per phospholipid molecule, for both DPPC and DPPG. This is in sharp contrast to the results obtained for chitosan in the subphase. As also indicated in Figure 5, chitosan causes a major decrease in the maximum in-plane elasticity, especially for the DPPG monolayer, with a shift toward larger areas per molecule of the maximum. (The

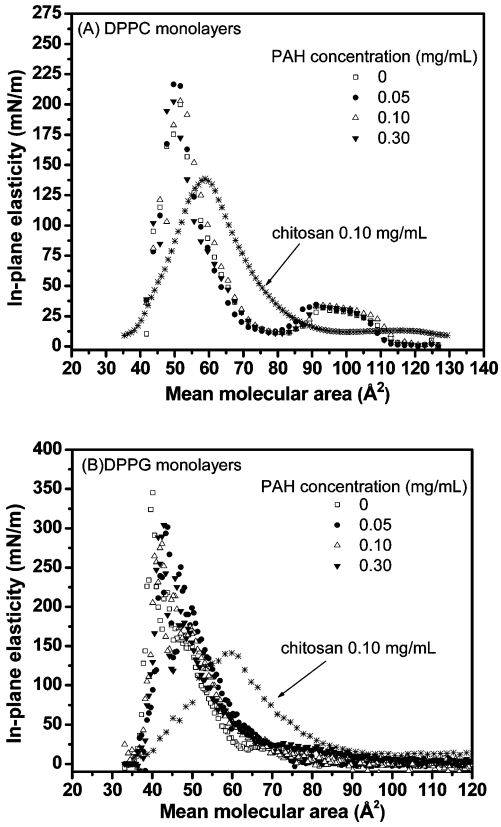

FIGURE 5. In-plane elasticity for (A) DPPC and (B) DPPG monolayers formed over pure Theorell buffer and solutions of PAH and chitosan in the same buffer. The concentrations of PAH and chitosan are indicated in the inset.

peak at 90 Å^2 for DPPC is due to the phase transition from the liquid-expanded to the liquid-condensed states.) The decrease in the in-plane elasticity probably results from the stronger interactions between the phospholipid polar heads and chitosan, in addition to the interpenetration of chitosan which affects the ordering of Langmuir and LB films (14, 15). Of particular relevance for the biological implications, chitosan reduces the monolayer elasticity significantly at a surface pressure believed to correspond to the lateral pressure of a cell membrane (in a biological membrane the packing is similar to that of a phospholipid Langmuir monolayer with surface pressure of 30−35 mN/m (26)), whereas PAH has a negligible effect.

Taking the results above together, it is clear that chitosan has a much more pronounced effect on the phospholipid monolayers, even though PAH is also cationic and bearing the same ionizable group (amine). Therefore, the action of chitosan should not be entirely attributed to the electrostatic nature of the interaction with model membranes; other forces should be involved.

We now resort to a characterization technique, namely the surface potential, which depends strongly on the charge of the monolayers or incorporated in the subphase (27–29). The measured surface potential, ΔV, can be related to the dipole moment of the molecules and the contribution from the double layer, as follows

$$\Delta V = \frac{1}{A\varepsilon_0}\left(\frac{\mu_1}{\varepsilon_1} + \frac{\mu_2}{\varepsilon_2} + \frac{\mu_3}{\varepsilon_3}\right) + \Psi_0 \qquad (1)$$

where A is the area per molecule, ε_0 is the vacuum permittivity, μ_1/ε_1 is the contribution from the reorientation of water molecules due to the presence of the monolayer, μ_2 and μ_3 are the normal dipole moment components from the headgroups and tails, respectively, and ε_2 and ε_3 are the corresponding effective dielectric constants of the media surrounding the headgroups and tails. Ψ_0 is the double-layer potential for charged monolayers. The adsorption of a polyelectrolyte on a phospholipid monolayer should cause a large change in surface potential, either by modifying the contribution from the double layer of charged phospholipids such as DPPG or by inducing a double layer for a zwitterionic phospholipid owing to the adsorption of charged species. Obviously, other changes in surface potential may arise, as the contributions from the reorientation of water molecules and even the packing of the molecules can be affected. Unfortunately, for a complex system such as the phospholipid monolayers interacting with PAH or chitosan one cannot analyze the surface potentials quantitatively. Nevertheless, the electrostatic effects should predominate (see the importance of surface charges in refs 9, 30).

As expected, the incorporation of the positively charged PAH increased the surface potential of DPPC and DPPG monolayers, with an overall change in the whole isotherms (see Figure S1 in the Supporting Information) and an increased effect with increasing concentration. The exception was again the 0.30 mg/mL concentration of PAH for the DPPG monolayer. Because this latter concentration is outside the range used for chitosan, we did not pursue this effect further. Nevertheless, it is thought that this may be due to the conformation adopted by the polymer and/or change in viscosity at this higher concentration, which in turn may affect ion pairing and orientation of the dipole moments.

As Figure 6 shows, the changes in the maximum surface potential induced by PAH at 0.10 mg/mL are even larger than those caused by chitosan, both for DPPC and DPPG. This trend may be ascribed to a higher charge density adsorbed at the air−water interface when PAH is used, which may be due to two factors: (i) the repeating unit of PAH is smaller than that of chitosan, thus leading to a higher charge density; (ii) under the conditions employed in our work, chitosan is believed to form a random coil in solution (31), whereas for PAH, one should expect a more extended chain, as in the molecularly thin films formed with the layer-by-layer (LbL) method (32, 33). The only case in which chitosan had a larger effect than PAH was in expanding the surface potential isotherm for DPPG, which should be expected because the monolayer itself was considerably expanded by chitosan, as indicated in the surface pressure isotherms.

The analysis of the surface potential results confirms the importance of the electrostatic interactions on the effects induced by chitosan on model membranes, as already suggested by many authors (12, 14, 15, 20). It indicated,

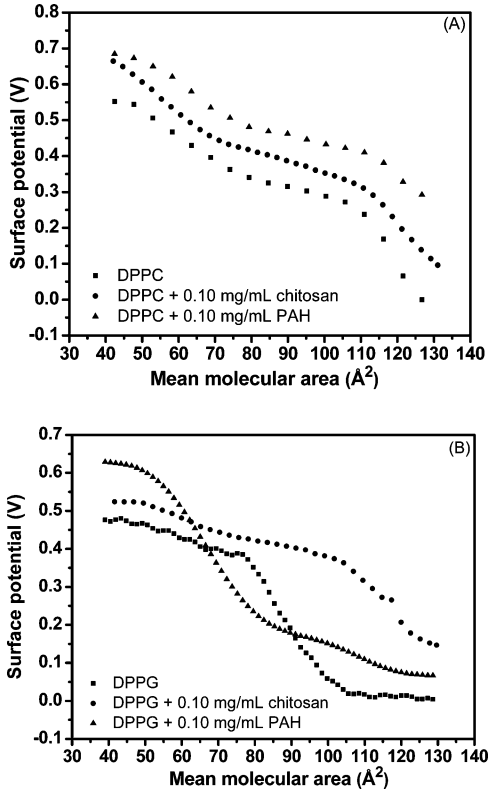

FIGURE 6. Surface potential isotherms for (A) DPPC and (B) DPPG monolayers formed on pure Theorell buffer (pH 3.0), PAH (0.10 mg/mL), and chitosan (0.10 mg/mL) solutions in the same buffer.

moreover, that PAH also causes large effects owing to electrostatic interactions, especially because the surface potentials are almost entirely dependent on the dipole moments and charges in the monolayers. The mechanical properties and packing may have an impact on the surface potential, but only in terms of possible changes in orientation of the dipoles. Therefore, if only electrostatic interactions mattered, PAH should have as large an effect on the model membranes as chitosan has. That the mechanical properties of the phospholipid monolayers—as investigated here with surface pressure isotherms and in-plane elasticity measurements—are more strongly affected by chitosan means that other interactions resulting from conformational as well as chemical composition differences are crucial to explain the activity of chitosan.

For the specific comparison between PAH and chitosan, the additional factors that may affect the interaction with the phospholipid monolayers are differences in molecular weight, in the conformation in solution and their ensuing steric effects, and in the charge density. As discussed above in connection with the surface potential measurements, charge density is believed to be higher for PAH, and therefore should not be able to account for the larger effects of chitosan on the mechanical properties of the monolayers. On the basis of our previous results and the contributions by others, namely Mohwald and collaborators (9, 30), the distinct conformations adopted by these polyelectrolytes appear to be a likely cause for the differences, as the penetration into the monolayers should involve hydrophobic interactions that depend on conformation. The reason why a coiled structure such as chitosan would be able to penetrate more easily into the monolayer, in comparison with the more linear PAH chain, is not known in detail, and is a subject under current investigation in our groups. Neither is it possible with the present data in the literature to identify the role of other functional groups of chitosan.

CONCLUSIONS

A direct comparison between the effects from chitosan and a positively charged polymer (PAH) served to demonstrate that electrostatic interactions are not sufficient to explain the action of chitosan on model membranes. The surface pressure and in-plane elasticity of DPPC and DPPG monolayers were much more affected by chitosan, even though PAH was also positively charged and with the same protonated group as chitosan. Nevertheless, the importance of electrostatic interactions was also confirmed with the results presented here, in two instances. First, larger effects from both chitosan and PAH were observed for the negatively charged DPPG, in comparison to DPPC. Second, in the surface potential data, for which electrical charges are the most important factor, the effects from PAH were even higher than those from chitosan because of the higher charge density of this polymer. The differences between chitosan and PAH point to the influence of other parameters, especially the conformation of these macromolecules in solution, but further studies are required for a complete understanding of its role and of possible effects from the hydroxyl groups and the sugar backbone of chitosan. Significant for the biological implications were the large changes in the monolayer elasticity induced by chitosan, which did not occur for PAH, as it is believed that activities such as the virus transfection may largely depend on the reduced elasticity of the membrane (34).

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Supporting Information Available: Surface potential-area isotherms for (A) DPPC and (B) DPPG monolayers over solutions of PAH (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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