Molecular View of the Interaction between ι-Carrageenan and a Phospholipid Film and Its Role in Enzyme Immobilization

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Proteins incorporated into phospholipid Langmuir−Blodgett (LB) films are a good model system for biomembranes and enzyme immobilization studies. The specific fluidity of biomembranes, an important requisite for enzymatic activity, is naturally controlled by varying phospholipid compositions. In a model system, instead, LB film fluidity may be varied by covering the top layer with different substances able to interact simultaneously with the phospholipid and the protein to be immobilized. In this study, we immobilized a carbohydrate rich *Neurospora crassa* alkaline phosphatase (NCAP) in monolayers of the sodium salt of dihexadecylphosphoric acid (DHP), a synthetic phospholipid that provides very condensed Langmuir films. The binding of NCAP to DHP Langmuir−Blodgett (LB) films was mediated by the anionic polysaccharide ι-carrageenan (ι-car). Combining results from surface isotherms and the quartz crystal microbalance technique, we concluded that the polysaccharide was essential to promote the interaction between DHP and NCAP and also to increase the fluidity of the film. An estimate of DHP:ι-car ratio within the film also revealed that the polysaccharide binds to DHP LB film in an extended conformation. Furthermore, the investigation of the polysaccharide conformation at molecular level, using sum-frequency vibrational spectroscopy (SFG), indicated a preferential conformation of the carrageenan molecules with the sulfate groups oriented toward the phospholipid monolayer, and both the hydroxyl and ether groups interacting preferentially with the protein. These results demonstrate how interfacial electric fields can reorient and induce conformational changes in macromolecules, which may significantly affect intermolecular interactions at interfaces. This detailed knowledge of the interaction mechanism between the enzyme and the LB film is relevant to design strategies for enzyme immobilization when orientation and fluidity properties of the film provided by the matrix are important to improve enzymatic activity.

Introduction

Several methodologies have been developed for immobilizing enzymes in solid supports for their use as biosensors or bioreactors.[1] Among them, the Langmuir−Blodgett (LB) technique enables the preparation of thin films built from organized multilayers of phospholipids. They are also a good model system for biomembranes and enzyme immobilization studies.[2−5] Nevertheless, it is well-known that different phospholipid compositions confer specific fluidities to the biomembrane, being an important requisite for enzymatic activity[6] and signal transduction.[7] Thus, the understanding of the organization of biomolecules in LB films may represent an important tool to study enzyme associations with biomimetic systems, as well as for the development of biosensors and/or bioreactors.

The LB technique is based on the vertical transfer of compressed monolayers of amphiphilic insoluble molecules,[8] from the air−water interface to solid substrates, the properties of these films being studied using a variety of techniques. Furthermore, packing modulation may be accomplished by the possibility of lateral compression of monolayers at air−liquid interface.

However, the LB technique does not allow great versatility in terms of surface pressure variation, since the transfer ratio and even the stability of films transferred at low surface pressure in general is not suitable, unless there is a specific interaction between successive layers. Hence, the LB film fluidity variation may be obtained by covering the top layer with different substances able to interact simultaneously with the phospholipid and the protein to be immobilized.

Several substances have been reported[9−12] to influence the phospholipid packing and monolayer compressibility. Cholesterol restricts the random movement of phospholipid head groups in some cases, altering the membrane elasticity.[13−15] Polysaccharides, often found in biomembranes, have a dramatic effect on membrane compressibility.[16−21] However, they may stabilize LB multilayer ending with hydrophilic groups, preventing the flip-flop[22] mechanism by establishing multiple bonds with the phospholipid head groups. Nevertheless, it is not a simple task obtaining information about which chemical groups of the polyelectrolyte bind to the phospholipids and which are available to immobilize the enzyme in nanostructures formed by a protein monolayer over a multilayer of phospholipid/polysaccharide. Due to their high sensitivity and symmetry-based selection rules,

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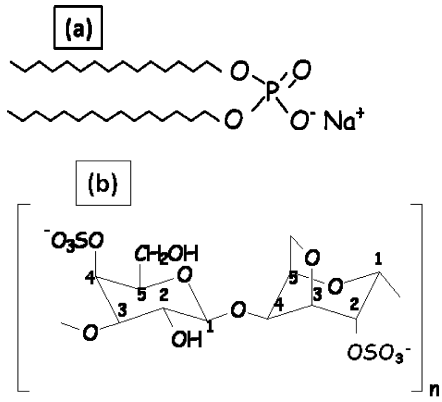

Figure 1. Structure of (a) DHP phospholipid and (b) ι-carrageenan disaccharide.

allowing us to distinguish centrosymmetric from noncentrosymmetric structures, nonlinear optical spectroscopic techniques[23] such as sum-frequency and second-harmonic generation have been used to investigate the conformation and orientation of molecules at interfaces.[24,25]

Carrageenans are a class of sulfated galactan polysaccharides that occurs in numerous seaweed species, mainly in the *Rhodophyceae* family. As cell wall constituents, their activity is related with the selective permeability of the cells. In this sense, they actuate as protector agents against dehydration in low tides.[26] Indeed, carrageenan is hygroscopic, playing an important role on spores and gametes liberation.[27−29] Carrageenans are classified into several subtypes according to their sulfate and 3,6-anhydro-D-galactose residues content, which can result in different conformations when in the presence of suitable electrolytes.[30]

In this work, the LB film was used as a simple membrane model system to study the effect of ι-carrageenan (ι-car) in the immobilization of the a conidial alkaline phosphatase from the filamentous fungus *Neurospora crassa*, which show a high carbohydrate content. To better understand the nature of molecular interactions occurring in this system, the quartz crystal microbalance (QCM) technique and vibrational spectroscopy by sum-frequency generation (SFG) were used.

Experimental Methods

Materials. Aqueous solutions were prepared using dust free Milli-Q water (surface tension of 72.8 mN m^{-1} and resistivity of 18.2 MΩ cm). Zinc acetate was purchased from Merck. Dihexadecylphosphoric acid, sodium salt (DHP) 99% (Figure 1a) was supplied by Sigma Chem. Co. Chloroform HPLC grade was from by J. T. Baker. All other reagents were of the highest purity commercially available. A purified extract of ι-carrageenan (Figure 1b), kindly supplied by Gelymar (Chile), was further purified by dialysis against water and converted into the Na^+ form using Dowex 50 WX4-100 ion-exchange resin (Sigma Chem. Co.), followed by rotoevaporation and freeze-drying. The efficiency of the procedure was controlled by flame spectrophotometry in a Shimadzu AA-680 model spectrometer. The molecular weight was determined by steric exclusion chromatography (SEC-MALLS) in a Waters HPLC (using four sequential Ultrahydrogel with exclusion limits of 4.105, 5.103, 7.106, and 8.104 g mol^{-1}), coupled to a multiple angle dynamic laser light scattering (Dawn-DSP-Wyatt Technology) and a differential refractometer (Waters, model 2410). Aqueous solutions in 0.1 mmol L^{-1} sodium nitrate were injected using a controlled flux of 0.6 mL min^{-1}. The molecular weight of ι-carrageenan was 2.5×10^6 g mol^{-1}.

Purification of *N. crassa* Conidial Alkaline Phosphatase (NCAP). The enzyme (NCAP) was purified from *N. crassa* wild type strain conidia.[31] Briefly, conidia from *N. crassa* grown in solid medium were harvested in H_2O and the suspension was stirred during 30 min at 0°. After removing the conidia, we purified the enzyme in solution in a single step by hydrophobic interaction chromatography in a phenyl−sepharose CL-4B column. Gel filtration and SDS-PAGE analyses suggested the native enzyme is a tetramer of identical subunits with M_r 145 kDa, p$I = 4.0 \pm 0.1$ and with an elevated carbohydrate content (ca. 40%).

Surface Pressure−Area (π−A) Isotherms. The monolayers at the air−liquid interface were obtained by spreading aliquots of DHP solutions prepared in chloroform and spread on three different subphases: pure water, 0.1 mmol L^{-1} zinc acetate, or 0.1 mmol L^{-1} zinc acetate and 100 ppm ι-car aqueous solution. The π−A isotherms were obtained at 24 ± 0.5 °C and pH 5.5, with a compression rate of 5 Å^2 $molecule^{-1}$ min^{-1} using an Insight Langmuir trough with total area of 216.2 cm^2.

LB Films. The quartz crystal microbalance (QCM) technique was used to determine the adsorbed mass per layer of phospholipid LB film, and also the adsorbed mass of carrageenan and enzyme on these films. Different strategies were used to immobilize the enzyme on solid supports, constituted by an initial three-layer LB film of DHP/Zn^{2+}. In all cases the films were deposited on a 0.662 cm^2 active area gold-covered quartz crystals (International Crystal Manufacturing, 10 MHz), previously cleaned with chloroform and ethanol, and dried under nitrogen flow. The oscillation frequencies (ICM Lever Oscilators, 10 MHz) were monitored (Lutron frequencymeter FC2700TCXO) until equilibrium was reached. Under specific conditions, changes in the oscillation frequencies are proportional to the adsorbed mass, according to the Sauerbrey[32] equation $F\Delta = (-0.26 \times 10^6 \times F_o^2 \times \Delta m)/A$, where $F\Delta$ and F_0 are the frequency change and the initial frequency (without coverage) given as Hz and MHz, respectively, Δm is the deposited mass (nanograms), and A is the electrode area (cm^2).

SFG Spectroscopy. The structure of DHP in Langmuir−Blodgett films and their interaction with ι-car was accessed by SFG vibrational spectroscopy, which is a nonlinear optical technique intrinsically specific to interfaces and sensitive to molecular conformation and orientation.[23] Briefly, two input laser beams, one in the visible range with a fixed frequency ω_{vis}, and another tunable in the mid-infrared with frequency ω_{IR}, overlap at an interface and generate an output beam at frequency $\omega_{SFG} = \omega_{vis} + \omega_{IR}$. The intensity of this sum-frequency (SF) signal is proportional to the square of the nonlinear susceptibility of the interface, $\chi_x^{(2)}(\omega_{SFG} = \omega_{vis} + \omega_{IR})$. As a second-order process, this technique is forbidden to media showing inversion symmetry but allowed to interfaces in which the inversion symmetry is broken. This is the reason why SFG spectroscopy can detect molecules at interfaces only, discriminating them against bulk ones. However, the net SF signal vanishes if there are molecules adsorb at an interface with random orientation. Conversely, if there is a substantial SF signal, it is possible to conclude that whether there are molecules having a net average orientation at the interface. When ω_{IR} is near the frequency of molecular vibrations, the SF output is resonantly enhanced, yielding a vibrational spectrum of the interface. Thus, in the present work the technique can monitor in a nonintrusive manner the structure and packing of phospholipid Langmuir−Blodgett films, and their interaction with polysaccharides. The SFG spectrometer (Ekspla) containing a

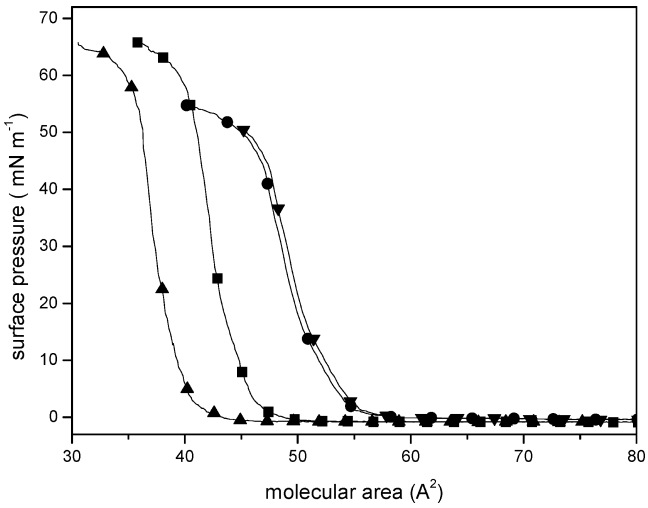

Figure 2. $\pi-A$ isotherms of DHP in different subphases: (■) pure water; (▲) 0.1 mmol L^{-1} Zn^{2+} ions; (●) 100 ppm ι-car + 0.1 mmol L^{-1} Zn^{2+}; (▼) 100 ppm ι-car + 0.1 mmol L^{-1} Zn^{2+} + 9 nmol L^{-1} NCAP.

pulsed Nd^{3+}:YAG laser provides a fundamental beam at 1064 nm (28 ps pulse duration, 20 Hz repetition rate). It pumps a harmonic unit which provides second and third harmonics (532 and 355 nm, respectively). The first is the visible beam that excites the sample (pulse energy of about 950 μJ, beam diameter around 1.5 mm). The latter is part of the fundamental beam pump, an OPA (optical parametric (oscillatory and amplifier) that generates a tunable infrared (IR) beam (pulse energy ~ 150 μJ, beam diameter ~ 1.0 mm) that overlaps the visible beam on the sample. The incidence angles are 51° and 60° for the IR and visible beams, respectively. The sum-frequency signal as a function of IR frequency is collected by a photomultiplier after spatial and spectral filtering. For each spectrum, data are collected with 100 shots/data point in 5 cm^{-1} increments in the 1000−1250 cm^{-1} range (S−O stretches) and in 3 cm^{-1} increments in the 2800−3000 cm^{-1} range (C−H stretches). In this work we used SSP (S-sum, S-visible and P-infrared) polarization combination.

For spectroscopic studies, the LB films were transferred to hydrophilic infrared-grade fused 3 mm silica substrates (Esco Products). For the CH resonance range, SFG spectra of films in contact with solutions (in situ) were recorded. For the OSO_3^- resonance range, in situ measurements were not possible since both the substrate and water are not transparent to the infrared beam. Thus, the experiments were then performed ex situ, immediatelly after immersion in the appropriate solution.

Results and Discussion

Effect of Zn^{2+} Ions and ι-Carrageenan in the Subphase on DHP Monolayers at the Air/Liquid Interface. The surface pressure (π)/area isotherms for DHP monolayers formed on different subphases are presented in Figure 2, and the general features of these isotherms are depicted in Table 1. Zinc ions were used to promote the interaction between the phosphate from the phospholipid polar headgroup of the phospholipid and ι-car sulfate group (Figure 1b).

As DHP yields highly condensed monolayers, the effects of Zn^{2+} are comparatively milder than those for other lipid monolayers.[33] Nevertheless, in the presence of Zn^{2+} ions the surface compressional modulus C_s^{-1}, defined as $(-\partial\pi/\partial \ln A)_T$ (see Table 1), reaches much higher values than those obtained when ι-car is also present in solution. Moreover, the concomitant addition of the polysaccharide and Zn^{2+} into the subphase causes an expansion of the monolayer, and the minimum area per molecule increaes from 40 $Å^2$ to almost 54 $Å^2$. We have also studied the influence of adding NCAP into the subphase containing ι-car and Zn^{2+} ions. In this condition, NCAP has no influence in the surface pressure (Figure 2). These results suggest that ι-car molecules are underneath the monolayer, interacting with DHP molecules. Contrarily, if NCAP molecules were close to the interface, they should interact with the ι-car layer bound to the phospholipid molecules, in such a way that the polysaccharide-phospholipid interaction is not disturbed.

TABLE 1: Surface Pressure (π)/Area Isotherm Features at 24 ± 0.5 °C for DHP Monolayers Formed at Different Subphases

Subphase for DHP monolayer	C_s^{-1}(max)/ (±2.0 mN m^{-1})	A_{min}/ (±0.8 $Å^2$)
pure water	795.4	46.7
Zn^{2+}	949.6	40.4
Zn^{2+} + ι-car	482.5	53.7
Zn^{2+} + ι-car + NCAP	473.1	54.0

Quantification of the Amount of DHP and ι-Carrageenan in LB Films. Assuming that Zn^{2+} can mediate the interaction between the DHP monolayer and ι-car, it is reasonable to expect that some amount of polysaccharide will be transferred along with the monolayer onto solid substrates by the LB technique. This may be attested and quantified, for example, by the QCM technique.

With this aim, DHP/Zn^{2+} monolayers were transferred onto gold covered QCM quartz crystals at a constant surface pressure of 30mN m^{-1}, forming Y-type LB films with 3-layers of DHP/Zn^{2+}, the hydrophobic chains of the phospholipids being exposed to the air in the third layer. A fourth layer was deposited by the immersion of these films through a DHP monolayer formed on different subphases: 0.1 mmol L^{-1} zinc acetate solution (subphase A), 100 ppm ι-car solution (subphase B), and 0.1 mmol L^{-1} zinc acetate mM) plus 100 ppm ι-car solution (subphase C). The films were rinsed for 10 min (to release ι-car molecules weakly attached to the surface), dried, and frequency changes were recorded. The experiments were carried out in triplicate. A schematic representation of these films, called hereafter type (A), (B), and (C), respectively, is shown in Figure 3. QCM results for the DHP/Zn^{2+} support layer and the different four−layer films are presented in Table 2.

The average mass per layer obtained for DHP/Zn^{2+} LB films reasonably agrees with that estimated from DHP/Zn^{2+} $\pi-A$ curve (Figure 2) assuming a DHP:Zn^{2+} 2:1 stoichiometry, resulting in a value of 162 ng. The difference between estimated and experimental values may be attributed to solvation water carried together with the ion. Further, the mass value of the last layer of B-type films corresponded to 153 ng; apparently, the difference from A-type films may be attributed to the absence of Zn^{2+}.

In contrast, the fourth layer of the C-type LB films presented a huge mass increase when compared to the value for A films, even considering the removal of about 15% of the mass after rinsing. This difference, thus, should correspond to ι-carrageenan effectively interacting with DHP. Considering the mass per disaccharide unity of ι-carrageenan, an amount of around 0.27 nmol of the disaccharide remains bound to the LB film and corresponds to an approximate 1:1 of phospholipid:disaccharide ratio at the interface. This result is indicative that Zn^{2+} ions might promote the binding of the anionic polyelectrolyte to the negatively charged phospholipid LB film. Further, it seems likely

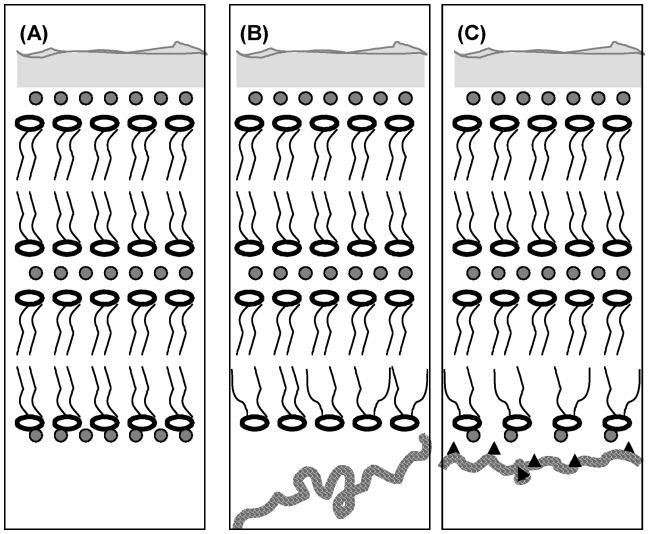

Figure 3. Schematic representation of DHP LB films A, B, and C with the fourth layer deposited from the following subphases: (A) 0.1 mmol L^{-1} zinc acetate solution; (B) 100 ppm ι-carrageenan solution; (C) 0.1 mmol L^{-1} zinc acetate plus 100 ppm ι-carrageenan solution. Filled circles correspond to Zn^{2+} ions; curled lines represent ι-car and arrows indicate the most probable orientations of ι-car sulfate groups for the bounded chain, after SFG results.

TABLE 2: QCM Results for DHP Langmuir−Blodgett Films

layer type	average mass (ng) per layer
[1]: DHP/Zn^{2+}	177.9 ± 1.5
[2]: DHP/water	153.0 ± 1.5
[3]: 4th Layer DHP/Zn^{2+} on ι-car	302.0 ± 1.8
[4]: [3] after rinse	260.7 ± 3.2
[5]: 4th Layer DHP on ι-car and NCAP.	198.9 ± 1.6

[a] Monolayers were transferred at 30 mN m^{-1}, 24 ± 1 °C, and dipping speed of 0.33 mm s^{-1}.

that in this condition the polyelectrolyte layer presents an extended conformation, with a quite limited number of loops.

Immobilization of NCAP on DHP LB Films. Two methodologies were tested to immobilize the enzyme, both having as a first step the formation of a three layer-LB film of DHP/Zn^{2+}. In one of them, the fourth DHP layer was deposited from a subphase containing 100 ppm ι-carrageenan plus 9 nmol L^{-1} NCAP, but not Zn^{2+}, aiming to transfer the polyelectrolyte and the enzyme simultaneously with the phospholipid monolayer. The resulting film was then rinsed and dried. The deposited mass was determined as 198.9 ± 1.1 ng (see Table 2), only 46 ng larger than that of a DHP/water layer, indicating that the amount of ι-car and NCAP transferred together with DHP is not very significant. In this case, rather than making a bridge between phospholipid and enzyme, the polysaccharide apparently interacts preferentially with the enzyme in the bulk. In this case, rather than making a bridge between phospholipid and enzyme, the polysaccharide apparently interacts preferentially with the enzyme in the bulk.

In an alternative procedure, a C-type LB film was immersed in a 9 nmol L^{-1} NCAP aqueous solution. Film withdrawal was carried out after a 20 min interval; the film was rinsed and dried, and frequency changes were measured. Finally, the film was immersed again in the NCAP solution for additional 20 min interval. The results shown in Figure 4 represent the kinetics of NCAP adsorption on a C-type LB film. It may be observed that the adsorbed mass increases continuously with time,

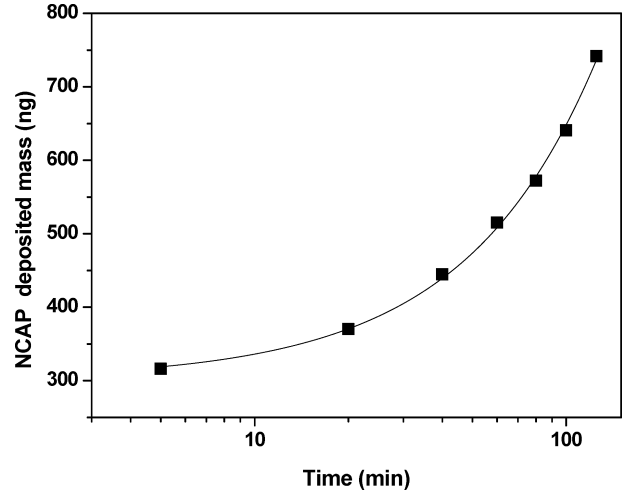

Figure 4. Kinetics of adsorption of NCAP on DHP/Zn^{2+}/ι-car film. The measurements were carried out discontinuously by sucessive immersions of a film during adequate time intervals, with posterior withdrawal and drying.

reaching a surface density of about 1.12 μg cm^{-2} after two hours, but no tendency to saturation is detected.

Considering that the molecular weight of the enzyme of 110 kDa, this adsorbed mass corresponds to an average molecular area per NCAP molecule of 21 nm^2. Although structural data for NCAP is not yet available, data from the mammalian placental alkaline phosphatase solved recently[34] allow us to consider NCAP as an ellipsoid measuring 10 × 5 × 5 nm, which yields limiting areas occupied by one enzyme molecule of 19.6 and 39.3 nm^2, depending on its orientation being perpendicular or parallel to the interface. Further, both the shape of adsorption curves and the estimated area occupied per molecule suggest that in the experimental conditions used, a NCAP multilayer type adsorption is occurring. In a control experiment, NCAP adsorption was carried out on an A-type film with all other conditions kept constant. In this case, the frequency change after 60 min was of about 49 ng, corresponding to 15% only of that measured after 5 min for the C-type film. Therefore, in the absence of ι-car, the adsorption of NCAP is almost negligible, indicating that the polysaccharide is essential for transferring the enzyme to the phospholipid LB film.

SFG Studies. Data from air/liquid interface (Table 1 and Figure 2) and QCM measurements (Table 2) suggest that the polysaccharide interacts with the DHP LB film only in the presence of divalent ions, and the adsorbed ι-carrageenan molecules enable the immobilization of NCAP. As this protein is rich in carbohydrate and it is above its p*I*, we envision two possibilities for its interaction with ι-carrageenan: by means of hydrogen bonding formation or through sulfate groups mediated by divalent cations. To investigate these two possibilities and the existence of a preferential orientation of the polysaccharide for enzyme immobilization, SFG studies were carried out.

Figure 5 shows SFG spectra in the region of sulfate stretches for all three types of films. The absence of SFG signal for the A-type film plays the role of a control, since this film has no OSO_3^- groups. Interestingly, the same behavior is observed for the B-type film, which is analogous to the C-type, but containing the fourth layer prepared in the absence of Zn^{2+}. This result could be interpreted in two ways: (i) the ι-carrageenan is adsorbed onto the phospholipid LB film, but having a random conformation, leading to isotropic orientation of sulfate groups, thus resulting in a vanishing SFG contribution, or (ii) the polysaccharide is not able to interact with the phospholipid

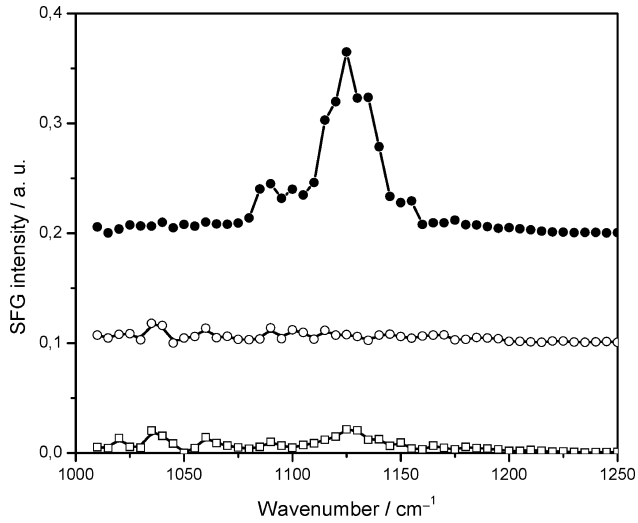

Figure 5. SFG spectra in the SO stretch region for films: (○) A-type (four identical DHP/Zn^{2+} layers); (○) B-type (three DHP/Zn^{2+} layers and 4th DHP deposited from ι-car subphase in the absence of zinc ions); (●) C-type (three DHP/Zn^{2+} layers and 4th DHP/Zn^{2+} and ι-car).

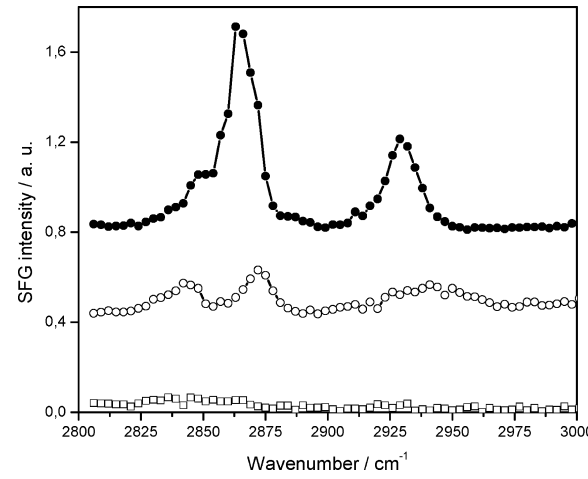

Figure 6. SFG spectra in CH stretch region for films: (□) A-type; (○) B-type; (●) C-type.

monolayer in the absence of Zn^{2+}, leading to a negligible adsorption of ι-carrageenan. This last possibility agrees with QCM results (see Table 2, layer [5]) and it is more plausible due to electrostatic reasons, since both the polysaccharide and phospholipid are negatively charged.

The C-type film exhibits a strong peak around ∼1125 cm^{-1} and a weaker one around ∼1090 cm^{-1}, as shown in Figure 5. A slight shoulder may also be observed at ∼1155 cm^{-1}. The assignment of these peaks is not straightforward and a detailed discussion is found in the Supporting Information. From literature, FTIR and FT-Raman spectroscopy studies show the presence of very strong absorption bands in the 1210−1260 cm^{-1} region for all types of carrageenans (attributed to S═O of sulfate esters) and a dual peak in the region 1010−1080 cm^{-1} attributed to the glycosidic linkage).[35] For ι-carrageenan, the peak in the region ∼1100 cm^{-1} is particularly intense, surpassing that attributed to the glycosidic linkage, most likely due to the presence of two sulfate groups per disaccharide in the macromolecule.[36] On the basis of literature data and calculation presented in Supporting Information of this paper, we attribute the observed peaks to the O═S═O symmetric stretch of the ester sulfate groups (−OSO_3^-) coupled to skeletal vibrations of the polysaccharide (C−C, C−O, and glicosidic linkage −O−). The presence of these peaks implies that the ester sulfate groups have a net average orientation in the adsorbed polysaccharide layer for the C-type film, pointing (on average) either toward or away from the phospholipid layer. Since the QCM results show that ι-carrageenan only adsorbs when Zn^{2+} ions are present in the subphase, the sulfate groups most likely point toward the phospholipid layer, with the electrostatic interaction between DHP and the polysaccharide mediated by the divalent ions.

To verify the ordering of the films and to establish the conformation of the adsorbed carrageenan, the CH stretch region was studied as well. Figure 6 shows the SFG spectra for the same films cited above but recorded in situ, that is, with the LB films in contact with the solution used as subphase for the last layer.

Analogous to Figure 5, SFG spectra for A-type film also vanishes in this region. This is due to the symmetry of the film formed by the transfer of four identical lipid/Zn^{2+} LB monolayers, resulting in a centrosymmetric arrangement of lipid tails.

For the B-type film, peaks around 2872 and 2944 cm^{-1} due to methyl groups[37] of the aliphatic hydrocarbon chain of DHP were observed, which are attributed to the CH_3 symmetric stretch (r^+) and to its Fermi resonance with the overtone of CH_3 symmetric bending (r^+_{FR}), respectively. The band around ∼2845 cm^{-1} is assigned to the symmetric stretch of methylene groups (d^+),[38] and it is only observed in the presence of gauche defects in the alkyl chains, breaking the local symmetry for the arrangement of CH_2 groups.[39] The presence of these peaks is explained by a break in the symmetry caused by the absence of Zn^{2+} in the last layer. This observation is in agreement with the isotherms for DHP on a subphase containing Zn^{2+} and on pure water. We have already mentioned that in the presence of Zn^{2+} ions, the monolayer is more compact, since the cations screen the electrostatic repulsion among the anionic lipid head groups. Without Zn^{2+} ions, the DHP monolayer is more expanded, leading to the occurrence of gauche defects, so that the contribution of methylene groups (d^+) to the SFG spectrum becomes significant. Therefore, the peaks in SFG spectrum of B-type films are not related to some modification promoted by ι-carrageenan, but to the different packing of the last phospholipid layer compared to that in A-type films, as illustrated in Figure 3. Combining the QCM results that show a negligilbe adsorption of the polysaccharide on theses films and SFG results of Figures 5 and 6, we conclude that the presence of carrageenan in the subphase does not affect the film structure, in the sense that no binding occurs.

For the C-type film, formed with the last DHP layer transferred from a subphase containing ι-carrageenan and zinc ions, we observe a significant change in the SFG spectrum, with main peaks at ∼2845, ∼2863, and ∼2929 cm^{-1}. A slight shoulder at ∼ 2872 cm^{-1} may also be noted. The peak at 2845 cm^{-1} and the shoulder at 2872 cm^{-1} (obscured by the pronounced peak at 2863 cm^{-1}) coincide with those observed for the B-type film and result from a different packing density of the last DHP layer, leading to gauche defects in its alkyl chains. As evidenced by the $\pi-A$ isotherms, in the presence of zinc ions ι-carrageenan promotes an expansion of the DHP monolayer, and at a surface pressure of 30 mN m^{-1} the molecular area per DHP molecule is higher than that observed for a DHP monolayer formed either on pure water or on zinc acetate solution. Therefore, just as in B-type films, the last layer of C-type LB films presents a more expanded packing of the lipid molecules, resulting in a more disordered conformation of their alkyl chains. The peak at 2863 cm^{-1} has been observed

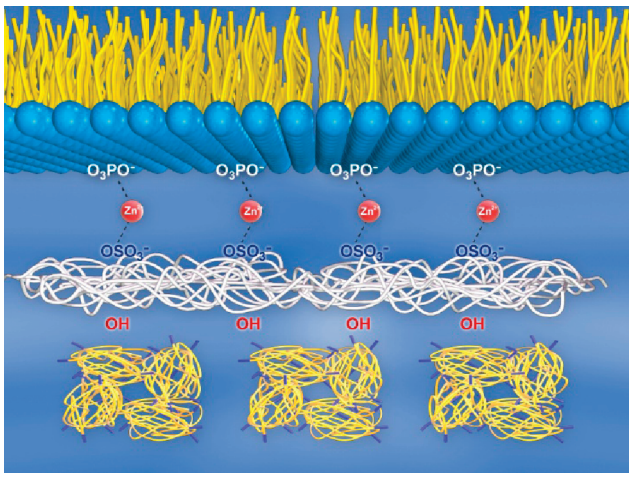

Figure 7. Model for NCAP and phospholipid interaction mediated by ι-carrageenan.

previously in the SFG spectra of lipids[40] and was assigned to the symmetric stretch of the CH_2 group adjacent to the terminal CH_3 group (d_{adj}^+). However, this assignment is not very likely in our case because this resonance should be quite weak in comparison to the CH_3 peak (r^+).[41] Alternatively, this peak at 2863 cm^{-1} is characteristic of saccharides.[42] This resonance and the one at 2929 cm^{-1} are typical of the symmetric stretch of CH_2 groups bound to O in ether compounds[43] (more specifically $-CH_2-OH$ and $-CH_2-O-$, respectively), which can be found in the carrageenan molecule (Figure 1b). Thus, the appearance of these strong peaks indicates the presence of ι-carrageenan in the film with an ordered orientation of polysaccharide CH_2 groups. This is consistent with the SFG spectrum in Figure 5, which shows that the sulfate groups are also oriented in C-type films. Therefore, ι-carrageenan adopts a chain conformation with OSO_3^- groups pointing toward the DHP/Zn^{2+} layer and ether and hydroxyl groups preferentially exposed to the subphase. Such a conformation would be driven by the electrostatic interaction of sulfate groups and zinc ions adsorbed at the DHP layer.

Conclusion

On the basis of the results presented in this paper we propose a model for the interaction among phospholipids, Zn^{2+} ions, ι-carragenan, and NCAP, as illustrated in Figure 7. It is likely that the same mechanisms are involved in similar systems.

Zinc ions act as a "bridge" between two anionic species, phospholipids and carrageenan. The electrostatic interactions drive a conformational change of the polysaccharide, resulting in the exposure of sulfate groups oriented toward the lipids. The consequent orientation of the remaining part of the molecule results in the exposure of ether and hydroxyl groups toward the solution, making them available for hydrogen bonding with enzyme molecules.

The absence of interactions between the negatively charged enzyme and lipid headgroups mediated by zinc, confirmed by the negligible adsorption of NCAP on A-type films in the absence of ι-carrageenan, corroborates this model and emphasizes the prominent role played by this polysaccharide in mediating the adsoprtion of the enzyme onto the phospholipid layer, and additionally confering the film a higher fluidity. Nowadays, there is a great deal of evidence that carbohydrates play an important a role in a variety of biological processes.[44] In particular, glycosaminoglycans (GAGs), high molecular weight linear polysaccharides constituted of repeating disaccharide units usually rich in sulfate groups, are present on all animal cell surfaces and in the extracellular matrix. These polysaccharides assume extended structures in aqueous solutions due to their strong hydrophilic nature based on their extensive sulfation patterns and are known to bind and regulate a number of distinct proteins, including chemokines, cytokines, growth factors, morphogens, enzymes, or adhesion molecules.[45] Whereas the essential role of protein−GAGs interactions in the regulation of various physiological processes has been recognized for several decades, the molecular basis underlying such interactions is poorly known. The results described in this study demonstrate how interfacial electric fields can reorient and induce conformational changes in macromolecules, which may significantly affect their intermolecular interactions. The proposed model may constitute a valuable insight to investigate protein−GAG interactions and the resulting modulation of protein biological function, e.g., enzymatic activity. Future studies employing the deglycosylated form of NCAP may also contribute to the investigation of the importance of protein-bound carbohydrates for protein−polysaccharide interactions. Detailed knowledge of the interaction mechanism between NCAP and the LB film is also relevant to design strategies for enzyme immobilization when orientation and fluidity properties of the film provided by the matrix are important to improve enzymatic activity.

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Supporting Information Available: Detailed discussion of the assignment of ester sulfate stretches for ι-carrageenan, including a table of calculations of its vibrational spectrum and figures of optimized geometries and normal modes. This information is available free of charge via the Internet at http://pubs.acs.org.

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