Kinetic and Structural Study of the Interaction of Myelin Basic Protein with Dipalmitoylphosphatidylglycerol Layers

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ABSTRACT The interaction of myelin basic protein (MBP) with dipalmitoylphosphatidylglycerol films has been investigated by means of a microgravimetric gauge sensitive to the changes in load and structural modifications of the layer deposited onto its surface. Fourier transform infrared spectroscopy, circular dichroism, and x-ray diffraction have confirmed protein uptake by the lipid phase along with a global disordering effect onto the lipid alkyl chains and have shown a temporal evolution of the structure of water penetrating the lipid phase together with the protein. These effects are clearly related to the temporal variation of the microgravimetric gauge signal. Finally, measurements carried out on pre-annealed samples point out the role of mesoscopic morphology in determining the pathways through which MBP penetrates the lipid multilayer. The results obtained in our model system could be useful in clarifying the mechanisms of the myelinating and demyelinating processes that take place in the natural membrane.

INTRODUCTION

Myelin basic protein (MBP) is a major protein of the myelin membrane in the central nervous system (~30% w/w) and is believed to be important for the compactness and the integrity of the membrane (Boggs and Moscarello, 1982; Stoffel, 1990). Despite the considerable amount of data that is present in the literature on the interaction of lipid-free MBP with model phospholipid membranes both in bulk (for a review see Smith, 1990) and in oriented lipid monolayers (Demel et al., 1973; Martenson, 1980; Maggio, 1997; Rivas et al., 1998) and multilayers deposited on a solid substrate (MacNaughan et al., 1985; Stuart, 1996, Haas et al., 1998), very little of it concerns the kinetic behavior of this interaction. To get information on this aspect should be important from a physiological point of view. In fact, it is well known that membrane surface is not a perfect, homogeneous layer, but, on the contrary, the presence of proteins and of different saturated and unsaturated lipids makes it extremely rough. Furthermore, phenomena like different phases coexistence, temperature jumps, etc., can create defects and discontinuities on the membrane surface, which could influence the organization and function of the membrane proteins. Therefore, model experiments could be useful in clarifying the mechanisms of the myelinating and demyelinating processes, mainly by studying the role of various effectors (e.g., metal ions) on the interaction mechanisms and kinetics of MBP on mono- and multilayers.

In the investigation of these phenomena, the use of bulk membrane models like vesicles is strongly limited by the fact that MBP causes vesicle fusion and sedimentation (Maggio et al., 1989). Indeed, addressing these problems requires the use of special techniques for phospholipid multilayer formation, such as the Langmuir–Blodgett technique. However, the study of the interaction of MBP with model membranes has been so far limited only to that of MBP with the monolayer at the air–water interface and, eventually, to the investigation of multilayers of phospholipids that have already interacted with MBP as Langmuir films (Haas et al., 1998).

Probing the kinetics of the interaction between MBP and a model sheath requires one more step in the development of suitable experimental approaches because the floating monolayer does not seem to be a satisfactory enough model system for myelin, and it can be studied by using a limited set of experimental techniques (Mohwald, 1995). Therefore, we have developed an approach, based on a quartz crystal microbalance (QCM), which takes advantage of the possibility of transferring phospholipid multilayers onto a solid substrate while allowing the continuous, real-time measurement of the kinetics of the interaction of MBP solution with this lipid lamellar phase (Facci et al., 1999).

In our previous preliminary report (Facci et al., 1999), we focused on the demonstration of our technique. In this article, we extend the scope of the work to include characterization of the proteo-lipid complex by circular dichroism (CD) measurements and the microscopic structure by small-angle x-ray diffraction, and to discuss the biological significance of such results. We find a strong correlation between the spectroscopic and structural characterization and the behavior observed with the QCM technique. We have also investigated the role of defects in the mesoscopic structure of dipalmitoylphosphatidylglycerol (DPPG) layers on the adsorption of MBP by repeating our measurements on pre-annealed samples. We find that the presence of defects

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strongly facilitates the penetration of MBP into the multilayer phospholipid structure.

MATERIALS AND METHODS

MBP was isolated and purified from bovine brain according to the procedure of Deibler et al. (1972), dialyzed against pure water and prepared in working solution at a concentration of 3.10^{-5} M. DPPG was obtained from Sigma-Aldrich (Milano, Italy) and used without further purification. In this study, we have chosen, as a model system, a negatively charged phospholipid such as DPPG to ensure electrostatic interaction with MBP. All other chemicals used were analytical grade.

In mono- and multilayer studies, DPPG was dissolved in $CHCl_3$ and MetOH at a concentration of 1 mg/ml and used as spreading solution in typical amounts of 100 μl. Langmuir films of DPPG have been prepared in a commercial trough using a $ZnCl_2$ 10^{-4} M water subphase. Compression speed was set at 3600 mm^2/min and deposition surface pressure was chosen to be 35 mN/m (solid phase). Given the rigidity of the monolayer, we resorted to the Langmuir–Schaefer (horizontal lift) technique to transfer multilayers onto the solid substrate. Typically, from 10 to 40 layers were deposited in each experiment onto different substrates according to the different experimental requirements; namely, quartz crystal cuts with gold electrodes for QCM kinetic experiments, silicon plates for FTIR and x-ray diffraction, quartz slides for CD measurements, and CaF_2 windows for both UV and IR measurements.

Protein–lipid interaction kinetics was investigated by means of a microgravimetric transducer operating in liquid environment. AT-cut quartz crystal oscillators (typical resonance frequency 10 MHz) with gold electrodes (electrode surface area 38.5 mm^2), driven by a home-made digital driver, have been inserted in a special measuring chamber (volume 10 μl), which allows the exposure of only one electrode to protein solution. This special setup enables the transducer to operate also in a liquid environment without a significant decrease of its quality factor. The transducers were calibrated to allow the determination of the deposited mass (Facci et al. 1993); they were found to exhibit a mass sensitivity of 0.57 ng/Hz and a linearity up to several KHz shift. Experiments were performed at 25°C by exposing one electrode to protein solution and recording the variation of the resonance frequency as a function of time. The resonance frequency shift is generally connected to the mass-amount adsorption onto the lipid-coated electrode and to the variation of the viscoelastic properties of the layer itself (Kremer et al., 1996). Thus, we could simultaneously determine the quantity of mass adsorbed onto the multilayer structure and the effect of such adsorption on the mechanical properties of the resulting sample.

FTIR measurements were performed in the 3500–1500 cm^{-1} wavenumber range using a Jasco FT/IR 420 spectrophotometer on layers deposited onto 0.36-mm-thick silicon (100) plates with native oxide. The spectral resolution was 4 cm^{-1}. X-ray low-angle diffraction data were taken on the same samples. $Cu_{K\alpha}$ radiation has been used in θ-2θ experimental geometry. Spectra were acquired with 0.02° angular resolution. CD measurements were carried out using a Jasco J715 spectropolarimeter, in the 260–180-nm wavelength range, using 0.2-nm step resolution, 2-nm bandwidth, and 4-s response time. Twenty lipid layers were transferred onto the inner side of the flat lid of standard 0.1-mm quartz cuvettes and exposed to protein solution for 15 min. Measurements were performed at 25°C in N_2 atmosphere filling the cuvette with pure water.

Optical assessment of protein amount in the DPPG multilayers after incubation with MBP has been obtained by transferring 40 monolayers of DPPG onto a CaF_2 window. Absorption spectra were acquired both in the UV (200–400 nm) and IR (1200–3500 cm^{-1}) range. Assuming that the extinction coefficient at 278 nm is 10,000 $M^{-1}cm^{-1}$ (Cavatorta et al. 1994), an extinction coefficient at 1650 $cm^{-1}$of 33,755 $M^{-1}cm^{-1}$ can be estimated. Annealing of the lipid samples has been performed in air at 37°C for 60 min in an oven.

DISCUSSION AND RESULTS

Figure 1 reports the QCM signal corresponding to a typical experiment of MBP adsorption kinetics. The resulting trend appears to be characterized by a highly multiexponential behavior, which has been successfully fitted with a stretched exponential law (Richter et al., 1989) $A(1 - e^{-(t/\tau)^{\beta}})$ with a value of $\beta = 0.37$, $\tau = 159$ min, and $A = 215$ Hz. To separate the simple effect of mass adsorption from the effect on the viscoelastic properties, we have performed the experiment as follows. We exposed the DPPG multilayer to MBP solution for the first 15 min, after which time the solution was replaced by water for the rest of the measurement. It is remarkable that the functional behavior is described by the same law in the temporal regions both before and after the removal of protein solution. Furthermore, the trend of this curve is the same even if we incubate the multilayer with the protein solution for longer times. From the calibration of our quartz balance, we can deduce an upper limit for the mass adsorbed after the first 15 min of exposure to the protein solution, and obtain 45.6 ± 0.2 ng (as we shall demonstrate later, the actual amount of adsorbed protein is smaller, because mass adsorption is only one of the two contributions to the overall frequency shift we measure). For longer times, the signal continues to vary with the same functional law, without any kind of break. Such a remarkable result suggests that the signal variation in this experiment is connected in a nontrivial way to protein adsorption to the lipid layer (e.g., it does not depend only on the load caused by the mass adsorption). Moreover, the value of the characteristic time τ is significantly longer than that of exposure to protein solution, revealing that other mechanisms affecting the viscoelastic properties of the lipid–protein layer are active. These mechanisms must be connected

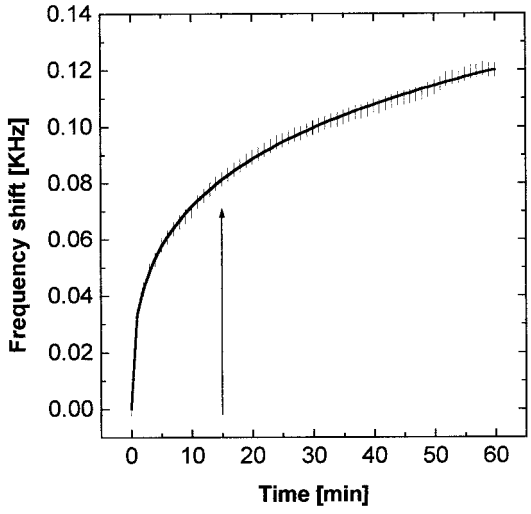

FIGURE 1 QCM adsorption kinetics of MBP solution (3×10^{-5} M) onto 10 LS layers of DPPG. See text for details. The arrow indicates the time at which protein solution has been replaced by pure water.

with protein interaction because the simple exposure of the lipid multilayer to water alone does not yield any detectable QCM signal variation in this time scale. Therefore, the origin of the observed variation has to be searched for in the modifications, which can occur in both protein and phospholipid layer structures due to adsorption of MBP.

It is known in literature that large structural modifications occur in MBP upon interaction with a lipid phase (Boggs and Moscarello, 1982); in particular, although MBP in water solution shows a largely random coil structure (Keniry and Smith, 1979), it gains an ordered secondary structure when it interacts with detergents, fatty acids, lipid micelles (Keniry and Smith, 1979), or Langmuir layers (Haas et al., 1998; Facci et al., 1999). Therefore, structuring dynamics, if present, could affect the QCM signal by varying the viscoelastic properties of the lipid–protein layer. To test this hypothesis, CD spectra on samples exposed to MBP solution were performed. Figure 2 reports the spectra obtained after 15 min MBP incubation and after a further 15-min exposure to water. Although these spectra show that protein has an ordered secondary structure, as revealed by the presence of the negative band at 207 nm, no temporal evolution appears in the spectra for at least a few hours. This fact shows that changes in protein secondary structure may contribute to the fast part of the curve of Fig. 1 but are already completed on the time scale of the signal variation shown in Fig. 1, and, hence, have no effect on the slower trend of the QCM signal. It is worth noting at this point that the shape of the CD spectra could be consistent with the presence of α-helices oriented with the axis perpendicular to the direction of the incident wave vector (i.e., parallel to the film plane) as demonstrated by de Jongh et al. (1994).

To gain insight on the parameters that are responsible for the temporal variation of the observed QCM signal, we have performed FTIR measurements on samples transferred onto silicon plates. Figure 3 reports the spectra resulting from this investigation. The experiment has been performed studying first the as-deposited DPPG multilayer (*curve A*), then the same sample after 15-min incubation in MBP solution (*curve B*), and after a further 15-min exposure to pure water (*curve C*). Clearly, marked bands in correspondence of the amide I and II regions (1700–1500 cm^{-1}) are present in curve B, revealing the presence of MBP in the sample. The intensity of these bands does not change even after incubating the sample for longer times. Thus, not only the protein structure, but also the protein uptake evolve on a faster time scale. From the measured extinction coefficient of the amide I peak (33,755 M^{-1} cm^{-1}), we are able to estimate the amount of protein adsorbed onto the multilayer after 15 min incubation, and find ~7.3 ng, which should be compared with the 45.6-ng upper limit estimated from the mass calibration of the microbalance. Thus, indeed, even within the first 15 min, the signal functional behavior is mainly determined by the protein-stimulated changes in the dynamic behavior of the multilayer. Furthermore, the C–H stretching bands (2800–3000 cm^{-1}) of the lipid chains appear to be quite affected, because both their position and full width at half maximum (FWHM) vary upon interaction with MBP (see Fig. 3, *inset*, and Table 1). In particular, the strong increase in the FWHM indicates a more disordered morphology of the alkyl chain as a result of the MBP uptake. Moreover, in spectra B and C, a broad band in the 3500–3000 cm^{-1} wavenumbers region is also present. In the spectrum B, this band shows a maximum at 3300 cm^{-1} and a pronounced shoulder at 3400 cm^{-1}. The band centered at 3300 cm^{-1} contains contributions due to the amide

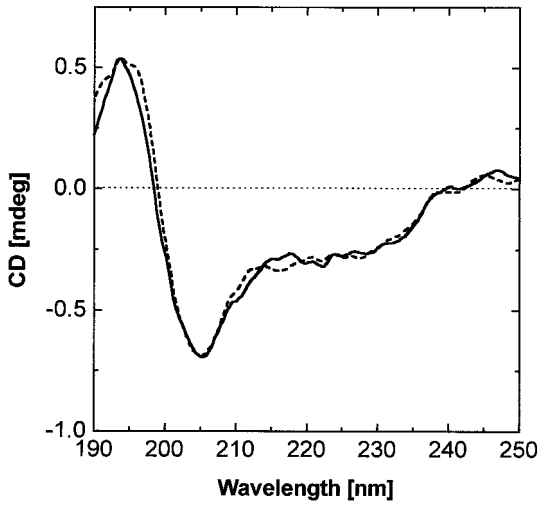

FIGURE 2 CD spectra of 10 LS layers of DPPG after exposure for 15 min to MBP solution (3×10^{-5} M) (*solid curve*) and after other 15 min exposure to pure water (*dashed curve*).

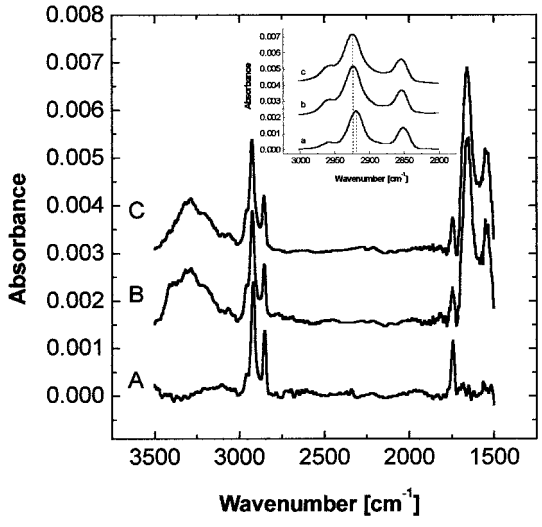

FIGURE 3 FTIR spectra of 10 LS layers of DPPG (*A*) before interaction with MBP, (*B*) after 15 min exposure to MBP solution (3×10^{-5} M), and (*C*) after 15 min more in pure water. The inset is a magnification of the C–H stretching region.

TABLE 1 Peak position and FWHM for antisymmetric C–H vibration in 10 LS DPPG layers upon interaction with MBP solution

Sample	$(C–H)_a$ Frequency [cm^{-1}]	$(C–H)_a$ FWHM [cm^{-1}]
DPPG	2917	18
after 15 min MBP	2921	25
+15 min H_2O	2923	27

Accuracy 1 cm^{-1}.

A of the protein and to the O–H stretching of water adsorbed to the polar heads of the lipids (Fringeli and Fringeli, 1979), whereas the shoulder at 3400 cm^{-1} could be attributed to bulk water that entered the film together with the protein. In fact, a considerable amount of water is believed to be present in the protein slab between the headgroups of charged lipids as proven by Haas et al. (1998) by neutron reflectivity measurements and in-situ exchange of the H_2O by D_2O. The shape of the water band is influenced by the different neighborhoods that water molecules can sense, depending on their interaction with proteins and polar headgroups inside the multilayer. Thus, it is important to separate the spectral changes due to water from those due to the protein uptake. For this reason, also in this case, experiments using deuterated water will be necessary to clarify the behavior of water in this process.

Curve C has been measured on the same sample but after a further 15-min incubation in pure water. It is possible to note that, whereas no changes are apparent in the amide I and II regions, the peak position and FWHM of the C–H stretching bands has evolved (Table 1). Moreover, the band in 3500–3000 cm^{-1} region appears quite different in shape, namely the shoulder at 3400 cm^{-1} has disappeared, consistent with a further structuring of water molecules inside the film. Thus, although, as stated, we cannot bring definite quantitative arguments for it, at least qualitatively, our data show an evolution of the local structure of the sample as evidenced by the water-related spectral changes, well after the protein had been removed from the solution.

We may also note that the protein penetrates the bulk of the multilayer sample as shown by the linear dependence of the intensity of amide I band on the number of deposited layers (Fig. 4). Therefore, this investigation identifies at least three parameters, which undergo a significant evolution upon incubation of the sample with MBP solution. The mass adsorption connected with the appearance of the band in the amides region is, of course, one of the phenomena responsible of QCM signal variation. However, the modifications in the C-H bands and the appearance and further evolution of the O–H bands show a temporal behavior consistent with that of QCM signal and could be related to the other mechanisms responsible for its slow variation, i.e., viscoelastic relaxation due to restructuring effects.

Thermal annealing at premelting temperature is known to generally remove defects in these films by increasing the domain size (see, e.g., Bourdieu et al., 1993). Therefore, performing adsorption experiments on pre-annealed samples can provide useful information on the role of defects in influencing the kinetic behavior of the interaction and on the mechanisms through which MBP can permeate the membrane.

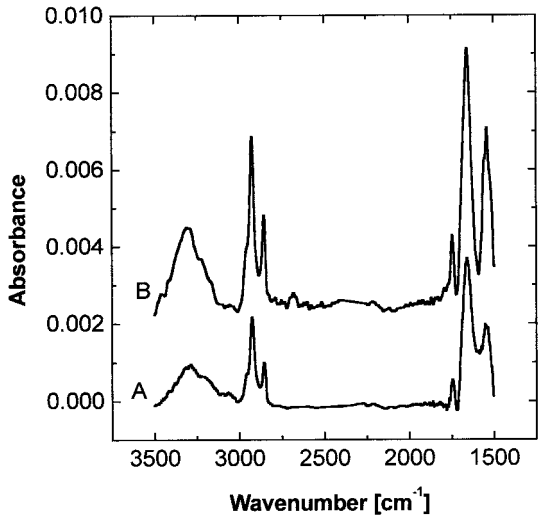

FIGURE 4 FTIR spectra of (*A*) 10 and (*B*) 20 LS layers of DPPG after 15 min exposure to MBP (3×10^{-5} M) and after 15 min more in pure water.

Therefore, QCM measurements on pre-annealed DPPG multilayers have been performed (Fig. 5). The analysis of these data shows that the saturation level of the curve is decreased, as well as the value of τ (15 min), whereas that of β appears to be increased (0.6). (The simultaneous variation of β and τ makes the time scale of the signal variation in Fig. 5 similar to that of Fig. 1, even though τ is an order

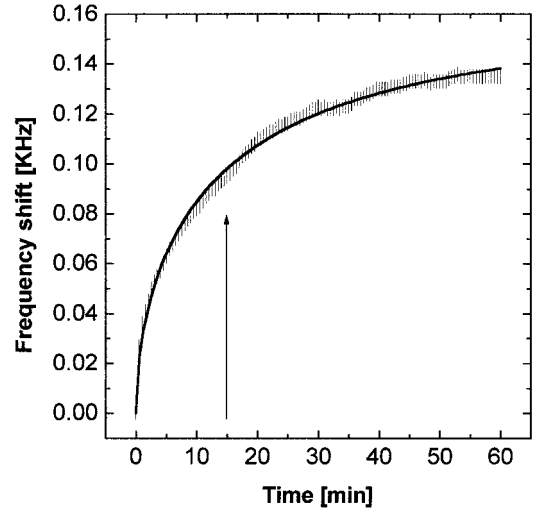

FIGURE 5 QCM adsorption kinetics of MBP solution (3×10^{-5} M) onto 10 LS pre-annealed layers of DPPG. See text for details. The arrow indicates the time at which the protein solution has been replaced by pure water.

of magnitude shorter. This is due to the peculiarities of the stretched exponential distribution: actually, τ is more accurately described as the first moment of the relaxation times distribution, which describes the complex relaxation, and which can be obtained in favorable cases by the inverse Laplace transform of the measured signal [Richter et al., 1989]). These results are consistent with a scenario in which MBP faces more difficulties in penetrating the multilayer due to its better mesoscopic order. Moreover, from the increase of β value and the correspondent decrease of τ of one order of magnitude, it is possible to argue that some mechanisms affecting viscoelastic properties, typically the slower ones, have been switched off.

FTIR spectroscopy performed on these samples (Fig. 6) shows, indeed, that the total amount of MBP that adsorbs to the pre-annealed lipid multilayer is decreased in comparison to the non-annealed sample This difference is consistent with that in the saturation value of the QCM curves. In particular, from the FTIR spectra, we estimate 4.2 ng for the total amount of protein contained in the multilayer, to be compared with 57 ng determined with the QCM technique. This is in agreement with our proposal that the signal variation of QCM is connected to relaxation in the sample. Moreover, both the position and FWHM of C–H stretching peaks do not show temporal evolution after 15 min incubation in MBP solution (Table 2) and the O–H stretching band does not appear, but only the band at 3300 cm^{-1} is present without any appreciable variation in time.

The results obtained measuring CD spectra on pre-annealed samples (Fig. 7) although confirming that no structural evolution takes place in the time scale of the experiment, show trends that are rather different from those measured onto fresh samples. In particular, the curves display a marked depression at 220 nm, consistent with a high content of ordered secondary structures, but do not show the expected positive lobe at 195 nm. A possible interpretation of this behavior could be correlated to a phenomenon of molecular aggregation, which should take place at the lipid layer surface because the molecules cannot penetrate the lipid phase through defects. However this must be confirmed by further experimentation. These results confirm that some molecular parameters that have been shown in the first set of experiments to have a temporal evolution compatible with that of the QCM signal have now consistently disappeared, confirming their role in the viscoelastic changes of the lipid–protein ensemble.

TABLE 2 Peak position and FWHM for antisymmetric C–H vibration in 10 LS DPPG pre-annealed layers upon interaction with MBP solution

Sample	$(C–H)_a$ Frequency [cm^{-1}]	$(C–H)_a$ FWHM [cm^{-1}]
annealed DPPG	2917	18
after 15 min MBP	2918	19
+15 min H_2O	2918	20

Accuracy 1 cm^{-1}.

More direct evidence on the lipid–protein film structure can be obtained by low-angle x-ray diffraction. Figure 8 reports the diffraction data obtained from a virgin 20-layer sample and from one exposed to MBP (*inset*). Clearly, the virgin layer shows a high degree of translational order in the z direction, which is essentially lost after exposure to MBP. However, some degree of local order is preserved, as evidenced from the shallow maximum in the MBP-exposed diffraction pattern. From its angular position, it is possible to evince that the film periodicity due to the lamellar structure (i.e., the position of the

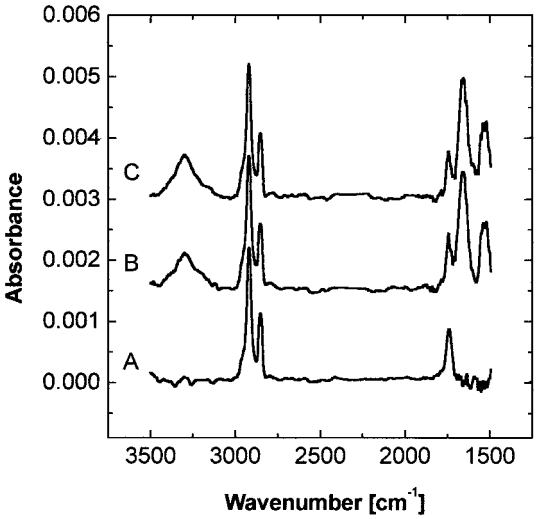

FIGURE 6 FTIR spectra of 10 pre-annealed LS layers of DPPG (*A*) before interaction with MBP, (*B*) after 15 min exposure to MBP, and (*C*) after 15 min more in pure water.

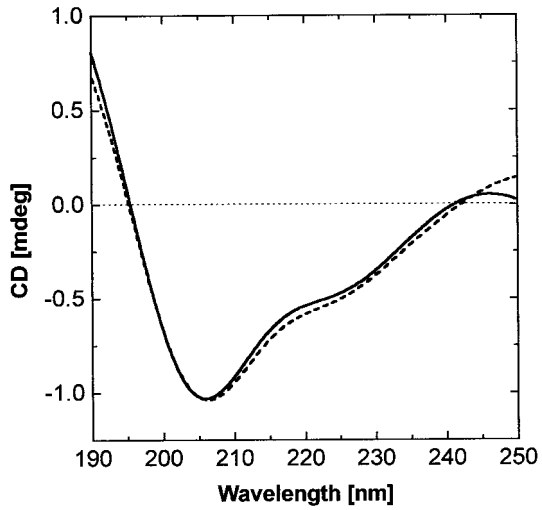

FIGURE 7 CD spectra of 10 pre-annealed LS layers of DPPG after exposure for 15 min to MBP solution (3×10^{-5} M) (*solid curve*) and after other 15 min exposure to pure water (*dashed curve*).

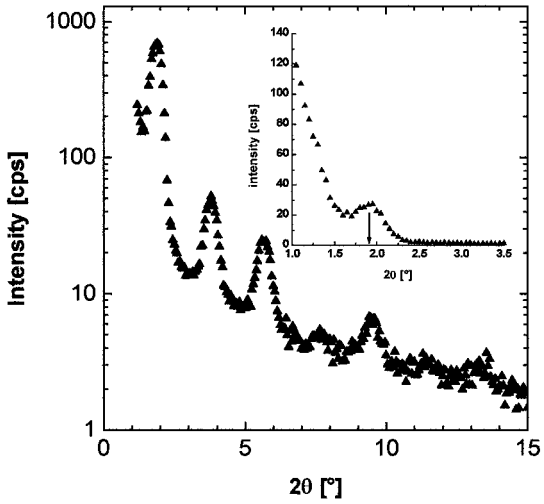

FIGURE 8 Low-angle part of the x-ray diffraction pattern of virgin 20-LS layers of DPPG. (*Inset*) The lowest angle part for the sample after exposure to MBP solution (3×10^{-5} M) for 15 min. The arrow identifies the angular position of the first maximum of the pattern of the virgin sample.

first-order Bragg peak at $2\theta = 1.89°$) is at least partially preserved and corresponds to a double-layer thickness of 4.8 nm, suggesting that no regular intercalation has taken place. However, the FWHM is increased, revealing a z-correlation length of about 24 nm, half of that of the as-deposited film. However, more precise measurements are needed to try to understand the nature of these effects.

CONCLUSIONS

The picture resulting from these studies shows that QCM technique can be successfully exploited to probe the kinetics of interaction of MBP with DPPG multilayers. Moreover, the role of film morphology at the mesoscopic level turns out to be crucial in determining the extent of protein uptake. Therefore, some hypothesis on the mechanisms ruling protein–lipid interaction can be made, based upon the comparative analysis of the various results. In fact, MBP permeates the membrane during interaction, as shown by FTIR spectra. Moreover, the protein resides, very likely, in the hydrophilic planes between polar headgroups, as suggested by the interpretation of CD spectra in terms of in-plane oriented α-helices. To get to these hydrophilic layers, MBP probably penetrates the membrane through defects, because adsorbed-protein amount decreases upon lipid layer annealing, and proteins show aggregation. The aforementioned considerations indicate the important conclusion that the protein adsorption interaction with the multilayer may be nonlinear, in the sense that, as the protein is adsorbed, it modifies the structure and dynamics of the multilayer; at the same time, the protein uptake itself is dependent on the mesoscopic structure of the multilayer.

This result is important also because it points out the role of defects as preferential pathways for MBP uptake by the lipid phase. As a consequence, it opens the question on the suitability of studying MBP–lipid layer interaction at the air–water interface where the film is much more defect-free due to the steady surface pressure value, and where it seems that only electrostatic interaction at the polar headgroups plays a significant role. Clearly this difference could be of biological significance: the membrane is much more similar to the partially disordered, mesomorphic, inhomogeneous system we have studied than to the homogenous, relatively well-ordered monolayer at the air–water interface.

More generally, the present results establish our QCM-based method as a sensitive, simple, and inexpensive way of monitoring mechanical and mass changes in complex biologically interesting systems. In the present case, the problem at hand was protein adsorption onto a lipid multilayer. However, many physico-chemical and biological processes can, in principle, be monitored by such technique, as long as they involve modifications of the mechanical (internal stress, viscoelastic, defect content) properties of the biological system.

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