Interaction of the Third Helix of Antennapedia Homeodomain with a Deposited Phospholipid Bilayer: A Neutron Reflectivity Structural Study

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Neutron reflectivity has been used to study the structure of deposited phospholipid bilayers in the gel phase and the modifications induced by the presence of a 16 amino acid peptide, the third helix of the Antennapedia homeodomain, *p-Antp*$_{43-58}$. The phospholipids were dipalmitoyl phosphatidylcholine (DPPC) and mixtures of DPPC with 10% mol/mol of the negatively charged dipalmitoyl phosphatidylserine (DPPS). They were deposited on silicon single crystals by using the Langmuir−Schaeffer technique. By pushing the resolution of neutron reflectivity measurements, the bilayer structure was determined at 1 Å precision. The thickness of the thin water layer between the substrate and the phospholipids was found to be 5 ± 1 Å, that of the headgroups 9 ± 1 Å and that of the chains 36 ± 1 Å. The deposited bilayer had the same roughness, 5 ± 1 Å, as the substrate itself. After peptide insertion, the thickness and roughness of DPPC bilayers did not change while the peptide appeared uniformly distributed in the interfacial region. In the presence of 10% DPPS the peptide was mainly detected in the headgroup regions and its insertion induced an increase of the bilayer roughness up to 12 ± 1 Å. No affinity between the peptide and the lipid hydrocarbon region was detected. Neutron reflectivity was shown once more to be a valuable technique for structural determination and with good potential for studies of relevance in biology.

Introduction

The growing interest in the incorporation of macromolecules in a cell is stimulated by the fact that it can be used for conveying drugs to a specific target in the cytoplasm and nucleus, while, at a fundamental level, it allows the study of the mechanism of transport across the barrier constituted by the lipid membrane. Progress in the field of molecular biology and in the chemistry of liposomes has led to the development of techniques for the introduction of activated molecules inside living cells, for example by linking them with other molecules normally internalized such as folate or bacterial toxins.[1] Unfortunately, those approaches suffer limitations related to the degradation of the active molecule, the modification of its chemical properties, or the disruption of the cell membrane.

Since the beginning of the 1990s, the idea of using a transmembrane vector, which would overcome most of the encountered problems, has become more and more realistic after the discovery that a short peptide,[2,3] the third helix of the Antennapedia homeodomain, *p-Antp*$_{43-58}$, and some of its analogues,[4,5] can translocate across neural cell membranes through a likely nonspecific interaction with the lipids of the membrane. Very little is known about this interaction. The experiments carried out on this system tend to exclude all classical mechanisms of cell insertion: the translocation across the plasma membrane does not require specific receptors or a helix conformation; internalization occurs both at 4 °C and at 37 °C and the mechanism is likely to be different from classical endocytosis.

Here we present the results of a neutron specular reflectivity study of phospholipid bilayers deposited in the gel phase on the surface of single crystals of silicon and the modifications induced to the structure by the incorporation of *p-Antp*$_{43-58}$, investigated with the membrane in contact with aqueous solutions. The effect of lipid head charges is studied. Biophysical techniques such as NMR,[6,7] surface infrared spectroscopy,[8,9] neutron diffraction,[10,11] and fluorescence microscopy[12] have been widely employed in the past to investigate lipid−peptide and peptide−peptide interactions. All these techniques imply

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the use of either phospholipid monolayers or stacked bilayers. Our approach involves the use of single phospholipid bilayers in an aqueous environment and a technique able to resolve details in the Ångstrom scale.

Neutron reflectivity[13,14] has already proved to be a powerful tool for the in situ characterization of condensed matter systems at interfaces. Neutrons are specularly reflected off an interface just like light or X-rays. Because of the short wavelengths available, neutron reflectivity has a resolution of a fraction of a nanometer. Furthermore, it is nondestructive and can be applied to buried interfaces which are not easily accessible to many other techniques. Since neutrons interact with atomic nuclei, isotopic substitution can be used for highlighting different parts of the interface (see below). Light elements such as carbon, hydrogen, nitrogen, oxygen, etc., are good scatterers, therefore neutrons are particularly suitable for the study of biological material.

Preliminary work on the interaction between the peptide in water and a monolayer of phospholipids deposited at the surface of water is described elsewhere.[15] Monolayers proved useful for the choice of lipids and concentration of peptide and also for the optimization of the technique of transfer of the lipids onto the solid substrate. Thermodynamic, ellipsometric, and spectroscopic measurements demonstrated the peptide surface-activity both under a lipid monolayer and in pure water (when injected in the bulk subphase, 25% of the peptide adsorbs at the water surface), its effect on lipid orientations, and the importance of lipid head charge in peptide−lipid interaction. At the air/water interface the peptide forms a stable monolayer up to ∼10 mN/m and under a quick compression can reach up to ∼50 mN/m. The monolayer is not at equilibrium: the surface pressure decreases as soon as compression is stopped. Another piece of work[16] presents a study on adsorbed phospholipid bilayers and double bilayers and their potential for use as model membranes; we refer to this paper for accurate details on the deposition techniques, neutron reflectivity measurements, data reduction, and analysis.

The work described below was confined to the lipid gel phase. The initial aim was to verify whether there was any affinity of the peptide with the hydrocarbon chains. As will be seen in the following paragraphs this is not the case. Our current research is directed toward fluid supported bilayers and their interaction with the peptide.

Principles of Neutron Reflectivity

Specular reflectivity, $R(Q)$, defined as the ratio between the reflected and the incoming intensities of a neutron beam, is measured as a function of the wave vector transfer, Q, perpendicular to the reflecting surface. $R(Q)$ is related to the scattering length density across the interface, $\rho(z)$, by the approximate relation:[13]

$$R(Q) \approx \frac{16\pi^2}{Q^2}|\rho(Q)|^2 \qquad (1)$$

where $\rho(Q)$ is the one-dimensional Fourier Transform of $\rho(z) = \Sigma_i \nu_i b_i$. ν_i is the number of nuclei per unit volume and b_i is the neutron scattering length, a quantity characteristic of atomic nuclei and varying irregularly through the periodic table. The composition profile is derived by model fitting using the more exact optical matrix formulation of the reflectivity.[17] The model consists of a series of boxes characterized by a scattering length density, $\rho(z)$ (from now on shortened as SLD), a thickness, t, and the interfacial roughness, σ, between two consecutive boxes. These parameters are varied until the optimum fit to the data is found. Although more than one model can be found for a given experimental curve, the number of possible models is greatly reduced by a prior knowledge of the system, which allows to define upper and lower limits of the parameters used, by the elimination of the physically meaningless parameters, and most importantly by the use of different isotopic contrasts. In fact, the use of a combination of hydrogenated and deuterated materials can substantially change the reflectivity curve of a system while maintaining the same chemical structure. All the reflectivity curves measured at different isotopic compositions of the same physical system must be described by the same model. Experience on this kind of systems and on similar ones[18,19] suggests that the measurement of reflectivity curves from three or more contrasts, combined with standard physical hypotheses, are necessary and sufficient for extracting a unique model of the interface.

Materials and Methods

Chemicals. The peptide *p-Antp*$_{43-58}$ is the fragment 43-58 of the third helix of the Antennapedia homeodomain. The 16-amino acid sequence is *Arg-Gln-Ile-Lys-Ile-Trp-Phe-Gln-Asn-Arg-Arg-Met-Lys-Trp-Lys-Lys*. The peptide has a high concentration of positively charged amino acids and p*I* equal to 12.78. It was purchased from Neosystem (purity >95% by HPLC) and counterions were replaced by Cl^- in a 0.1 N HCl solution. The peptide was then lyophilized for storage and purity was checked with mass spectrometry. It was dissolved in pure water before use. The phospholipids were DPPC and DPPS, both purchased from Avanti Polar Lipids. For DPPC both hydrogenated (h-DPPC) and perdeuterated (d_{75}-DPPC: in this compound the five hydrogens of the glycerol group are not replaced by deuterium) species were used. Fully hydrogenated and chain deuterated DPPS (h-DPPS and d_{62}-DPPS, respectively) were used. A mixture of DPPC and 10% mol/mol DPPS will be referred to as h-PCPS when lipids are hydrogenated and d-PCPS when they are deuterated. Phospholipids were dissolved in high purity chloroform (99.9%). The two substrates were single crystals of silicon (5 Å rms roughness) with dimensions $5 \times 5 \times 1$ cm^3 polished on one large face (111) by SESO, Aix-en-Provence. Single crystals of silicon are convenient substrates because they are transparent to neutrons and the beam can hit the interface from the solid side. Before each lipid deposition the surface of the blocks was cleaned by rinsing in chloroform, acetone, and ethanol, and made hydrophilic with a 30 min UV/ozone treatment.[20] High purity water (18 MΩcm, Elga) and D_2O (99% provided by the ILL) were used to prepare the contrast solutions for the reflectivity measurements. They were mixed in the volume ratios 0.62:0.38 to give SMW (silicon match water = water with the same SLD of silicon, 2.07×10^{-6}Å^{-2}, cfr. Table 1). Additional contrasts were used intermediate between those of silicon and D_2O, and indicated as XMW, meaning that the SLD is $\times 10^{-6}$ Å^{-2} (see Table 2). The measurements in the presence of the peptide were done in physiological phosphate buffered saline (PBS) solutions at pH

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Table 1. Properties of Materials Used

material	volume[a] (Å^3)	length[b] (Å)	b[c] (10^{-4} Å)	SLD[d] (10^{-6} Å^{-2})
Si	20		0.42	2.07
SiO_2	47		1.59	3.41
H_2O	30		−0.17	−0.56
D_2O	30		1.91	6.35
palmitoyl chains: $C_{30}H_{62}$	800	20.5	−3.24	−0.41
$C_{30}D_{62}$	800	20.5	61.31	7.66
heads PC: $C_{10}H_{18}O_8PN$	344	7.6	6.00	1.74
$C_{10}D_{13}H_5O_8PN$	344	7.6	19.61	5.70
heads PS: $C_8H_{11}O_{10}PN$	321		8.46	2.63
p-Ant$_{43-58}$ (in H_2O)	2702	24	44.42	1.64

[a] Molecular volumes calculated from densities for inorganics; from ref 34 for DPPC at 24 °C; from a simulation (WebLab ViewerPro software) for PS; sum of volumes of amino acids ref 24 for *p-Ant*$_{43-58}$; volumes are assumed to be independent of deuteration. [b] Length of the fully extended chain calculated from ref 35; thickness of PC heads from ref 36 peptide length corresponding to the α-helix conformation. [c] Scattering lengths calculated from ref 37 and ref 24. [d] Scattering length densities calculated by dividing the scattering lengths by the volume.

Table 2. Lipid Composition and Water Contrasts Used for the Reflectivity Measurements

	samples	water contrasts
1	2 silicon blocks	D_2O-4MW-SMW-H_2O
2	h-DPPC	D_2O-SMW-H_2O
3	d_{75}-DPPC	SMW-H_2O
4	h-PCPS	D_2O-4.5MW-SMW-H_2O
5	d-PCPS	D_2O-4.5MW-SMW-H_2O
6	h-DPPC + PEPTIDE	D_2O[a]-4MW[a] − SMW[a]
7	d_{75}-DPPC + PEPTIDE	5.3MW[a]-4.5MW[a]-SMW[a]-H_2O[a]
8	h-PCPS + PEPTIDE	D_2O[a]-4.5MW[a]-H_2O[a]
9	d-PCPS + PEPTIDE	D_2O[a]-4MW[a]-SMW[a]-H_2O[a]

[a] PBS solutions.

7.2 ± 0.1, that is pure water solutions containing 9 g/L NaCl, 13.6 g/L KH_2PO_4, 3.2 g/L NaOH. See Table 1 for the properties of the materials used.

Bilayer Deposition. The bilayers were deposited on the solid substrates at T = 24 °C with the Langmuir−Schaeffer technique.[21] After spreading a phospholipid monolayer on the water surface at a pressure < 0.1 mN/m, and allowing for the evaporation of the solvent, the monolayer was compressed to a surface pressure of 30 mN/m and the highly hydrophilic block was immersed in the subphase. By withdrawing the block from the water, at a speed of 5 mm/min, a monolayer of lipid was deposited on its surface. The solid substrate was then rotated by 90° and slowly reimmersed with the large face parallel to the water surface for the formation of the bilayer. The pressure was kept constant at all times. Stable and reproducible bilayers were obtained.[16]

The peptide-containing bilayers were prepared by first spreading the lipids on a PBS subphase (pressure < 0.1 mN/m) and then injecting a solution of the peptide corresponding to a final concentration in the bulk volume of 7.2×10^{-7} M. After waiting for about 30 min, to allow for the equilibration of the system, which was controlled by measuring the surface pressure in real time, the lipids plus peptide were compressed out of equilibrium to a surface pressure of 30 mN/m. The bilayer was deposited immediately afterward on the silicon substrate, already immersed in water, by using the same procedure as above.

Reflectivity Measurements and Analysis. Measurements were made at the silicon/water interface, the beam passing through 5 cm of silicon, at room temperature (~24 °C). They were performed on the D16 diffractometer at the Institut Laue-Langevin (ILL), specially modified for reflectivity measurements. Filtered monochromatic neutrons of wavelength 4.52 Å, with a wavelength spread $\Delta\lambda/\lambda \approx 0.01$, were collimated by two sets of slits located before the sample position. Neutrons specularly reflected by the sample, whose plane was oriented vertically, were detected by a two-dimensional ^{3}He detector placed at a 1 m distance. The reflected intensity was measured as a function of glancing angle of incidence, θ, by keeping the detector fixed and rotating the sample. By varying the slit apertures only twice along the measurement, $\Delta\theta$ was kept small and constant at each slit aperture ($\Delta\theta/\theta \leq 0.04$).

Table 3. Parameters Derived from Model Fitting the Reflectivity Profiles from the Protonated and Deuterated Phospholipid Bilayers

interfacial layer	thickness (Å)	% water	roughness (Å)
water	5 ± 1[a]	100[b]	5 ± 1[a]
outer1	9 ± 1	35 ± 10	5 ± 1
inner	36 ± 1	20 ± 5	6 ± 1
outer2	9 ± 1	40 ± 10	6 ± 1

[a] Error bars are statistic. [b] Parameter not fitted.

Table 4. Parameters Derived from Model Fitting the Reflectivity Profiles from the Protonated and Deuterated Phospholipid Bilayers + Peptide[a]

interfacial layer	t (Å)	SLD (10^{-6} Å^{-2})	% water	σ (Å)
water	4.5 ± 1.5[b]	—	100[c]	6 ± 1[b]
h-DPPC: outer1	9 ± 1	1.7 ± 0.5	45 ± 5	6 ± 1
inner	37 ± 1	0.2 ± 0.1	30 ± 3	6 ± 2
outer2	9 ± 1	1.7 ± 0.5	45 ± 10	7 ± 2
d_{75}-DPPC: outer1	9 ± 1	5.4 ± 0.5	40 ± 10	4 ± 2
inner	37 ± 1	5.2 ± 0.1	30 ± 5	4 ± 2
outer2	8 ± 1	5.4 ± 0.5	40 ± 10	4 ± 2
h-PCPS: outer1	13 ± 2	1.7 ± 0.4	40 ± 10	14 ± 2
inner	29 ± 2	−0.2 ± 0.2	15 ± 5	14 ± 2
outer2	13 ± 2	1.7 ± 0.4	45 ± 10	14 ± 2
d-PCPS: outer1	13 ± 2	4.6 ± 0.4	50 ± 10	10 ± 5
inner	29 ± 2	5.9 ± 0.3	20 ± 5	11 ± 3
outer2	13 ± 2	4.6 ± 0.4	50 ± 20	11 ± 3

[a] t = thickness; SLD = scattering length density; σ = roughness. [b] Error bars are statistic. [c] Parameter not fitted.

All measured reflectivity curves are summarized in Table 2. For each sample the measurement in D_2O was always the first in the set to be performed and it was repeated after measuring all the contrasts in order to ensure that during the rinsing and changing of contrast neither lipids nor peptide had been removed from the bilayer. Since the measured curves were identical within experimental error, we assumed that the structure at the interface remained unchanged.

Data were analyzed by model fitting using the programs AFIT[22] and WETDOC,[23] both allowing the simultaneous analysis of all data sets from the same sample in different water contrasts. As already mentioned, the main problem in reflectivity analysis is to find a model which fulfils two types of constraints: (i) fit all data, (ii) obey all physical hypotheses. If there are enough constraints there is only one such physically meaningful model.

The number of constraints was increased by providing more data to be fitted by the same model. Errors on parameters were calculated by varying these one after the other and by evaluating the χ^2 and visually inspecting the quality of the fit: by definition, a parameter value falls within the error bars if it simultaneously fits all the reflectivity curves measured for the same sample at all contrasts. The water content of each layer was determined independently from the variation of the box SLD in the different water contrasts. The SLD values given in Table 4 are the SLDs of the box nonaqueous fraction. These error bars are much smaller than when each curve is considered independently. This is a unique feature of neutrons, made possible by deuteration. Since

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this requires the preparation of different samples, or the rinsing of a sample with different subphases, the reproducibility of sample preparation was optimized and checked a posteriori. It was also assumed that the hydrogenated and deuterated lipid species behave identically at the interface and this was confirmed during data analysis. A common structure and composition was looked for both the h-DPPC and d_{75}-DPPC. The model found fitted equally well the h-PCPS and d-PCPS curves. The resulting 13 reflectivity curves (data from 2 to 5 in Table 2) were then fitted simultaneously and a very precise model of the bilayer alone was obtained. During the first trials of data fitting the outer layers of the membrane (headgroups) where found to be symmetric within experimental error both in terms of thickness and of SLD. This was valid for all curves in all contrasts, both in the presence and in the absence of peptide. In the final optimization of the fits the symmetry was therefore imposed in order to simplify the analysis.

The introduction of the peptide into the bilayer meant more free parameters. To preserve the precision of the analysis, the model of the bilayer alone was taken as the starting point and all parameters (thickness, roughness, water content, and SLD) were tuned in small steps. This procedure yielded the SLD of each box without any assumption on the proportion or the SLD of each of its components (see below). In addition to this local analysis, very different models (either physically expected models, or totally unexpected ones) were tried, but none of them gave good fits to the data. Here again, it was assumed, and then checked a posteriori, that deuteration did not affect the structure of the system. However, in contrast with the bilayer without peptide, a large difference was found between uncharged bilayers (h-DPPC + peptide and d_{75}-DPPC + peptide) and charged bilayers (h-PCPS + peptide and d-PCPS + peptide).

The SLD of each box was then analyzed in terms of composition. In principle, the respective volume fractions x, y, and z of lipid heads, chains and peptide in each box can be calculated by solving the following equation:

$$x\,\mathrm{SLD}_{heads} + y\,\mathrm{SLD}_{chains} + z\,\mathrm{SLD}_{peptide} = \mathrm{SLD}_{box} \qquad (2)$$

where SLD_{box} is the scattering length density of the box nonaqueous fraction experimentally determined. For one box (for instance, the outermost one in the charged bilayers with peptide) eq 2 is written as many times as there were available data with different lipid or subphase deuteration. The unknowns x, y, and z also have to obey volume constraints within each layer ($x + y + z = 1$) and between layers. If the set of linear equations in x, y, and z is redundant there are enough equations for a robust determination of their values.

As for $\mathrm{SLD}_{peptide}$, two approaches are possible: either to fix it to its predicted value, or to consider it as an unknown variable along with x, y, and z. Both methods rely on the assumption that $\mathrm{SLD}_{peptide}$ is the same in the inner and outer layers.

The calculation of the peptide SLD in different isotopic compositions of the solvent implies the knowledge of both its exact volume and the number of labile protons actually replaced by deuterium, that is those accessible to the solvent. Both quantities depend on the conformation of the peptide, which varies with its environment and fluctuates in time since the peptide is too short to have a secondary structure stabilized by hydrogen bonds.[15] When all the exchangeable protons of *p-Antp*$_{43-58}$ are replaced by deuterium in D_2O[24] and the volume is estimated by summing the volumes of all the amino acids present in the peptide, the calculated SLD is equal to 1.64×10^{-6}Å^{-2} in H_2O and 3.45×10^{-6}Å^{-2} in D_2O, linearly interpolated as $1.79 + 0.26 \times$ SLD-(solvent). It is possible that not all exchangeable protons are replaced; in that case the effect of the isotopic substitution of the peptide on the parameters used to fit the membrane falls into the error bars of the SLDs of the various layers and it is therefore negligible.

Results

The bare silicon substrates were characterized first, in terms of thickness and composition of the native oxide layer grown on the silicon surfaces. This was followed by the characterization of the lipid bilayers and finally of the peptide containing bilayers.

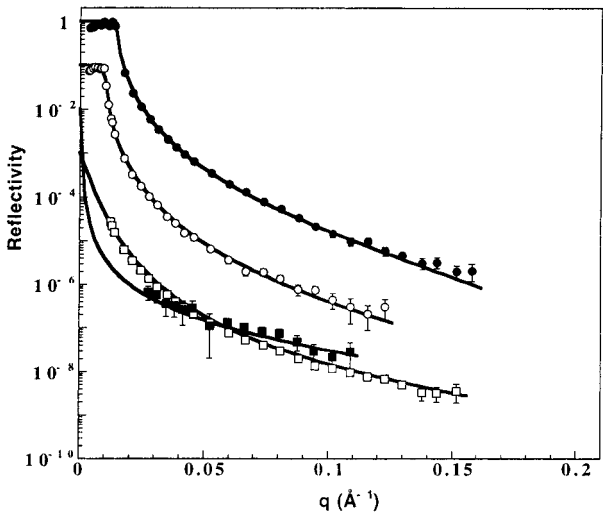

Figure 1. Neutron reflectivity profiles and fitted curves of the bare substrate in (●) D_2O, (○) 4MW, (■) SMW, and (□) H_2O. For clarity reflectivity from 4MW is divided by 10, from SMW by 100 and from H_2O by 1000. For the parameters used to fit the layer see text.

Bare Surface. We used two silicon blocks and measured the reflectivity in four water contrasts (see Table 2) for both blocks after they had been cleaned with a procedure including UV/ozone, the same used immediately before the deposition of the lipid bilayer.[20] For a given contrast, the reflectivity curves from the two blocks were indistinguishable within errors, as expected for identically treated samples. One set of four curves is shown in Figure 1, together with the curves calculated for a uniform single layer model (the silicon oxide layer) of thickness 12 ± 2 Å, roughness 5 ± 1 Å, and a scattering length density of 3.41×10^{-6} Å^{-2}, corresponding to 100% SiO_2.

Membrane Characterization. Both bilayers formed from pure DPPC and mixtures of DPPC with 10% DPPS (PCPS) were investigated in order to check the effect of negative head charges in the bilayer on the interaction with the peptide. All the contrasts measured (see Table 2) enabled fine details on the deposited bilayers to be obtained, since the contributions coming from the heads and the chains in the phospholipid region could be distinguished. The curves are shown in Figures 2 and 3 together with the fitted curves.

The parameters used to fit the curves simultaneously from all the contrasts are given in Table 3. The structure of the bilayer is the same both for pure DPPC and with 10% DPPS. As expected, the deuteration of the lipids did not affect the bilayer structure. A five box model was found to best fit the data. The first box is the oxide layer, already described above. A thin layer of water (5 ± 1 Å) between the oxide and the phospholipids is then detected. The following three boxes describe the deposited phospholipids. The outer boxes are well described by the theoretical SLD of the lipid headgroups given in Table 1, with the due corrections introduced when 10% protonated PS groups are present and with error bars of $\pm 0.2 \times 10^{-6}$ Å^{-2}. It is reasonable to assume that these two regions, adjacent to the solid substrate (outer 1 in Tables) and the bulk water (outer 2) consist of phosphatidylcholine and phosphatidylserine headgroups. The inner box can be described by the theoretical SLD in the case of hydrogenated chains while for the perdeuterated products it is found to be equal to $(7.2 \pm 0.2) \times 10^{-6}$ Å^{-2} to be compared with the theoretical

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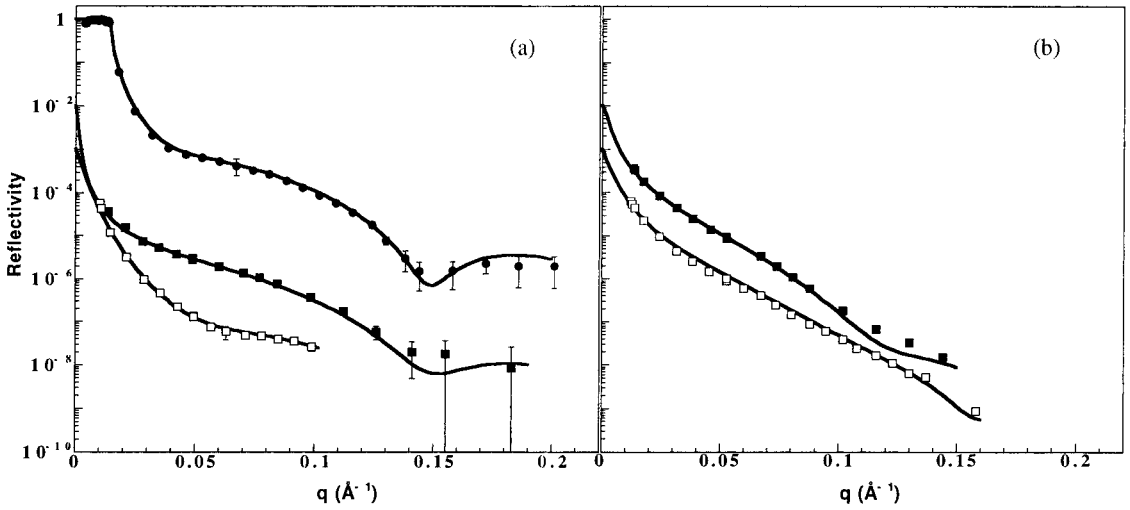

Figure 2. Neutron reflectivity profiles and fitted curves for the system (a) Si/SiO_2/h-DPPC/water in (●) D_2O, (■) SMW and (□) H_2O; and (b) Si/SiO_2/ d_{75}-DPPC/water in (■) SMW and (□) H_2O. For clarity reflectivity from SMW is divided by 100, and from H_2O by 1000. For the parameters used to fit the layer see Table 3

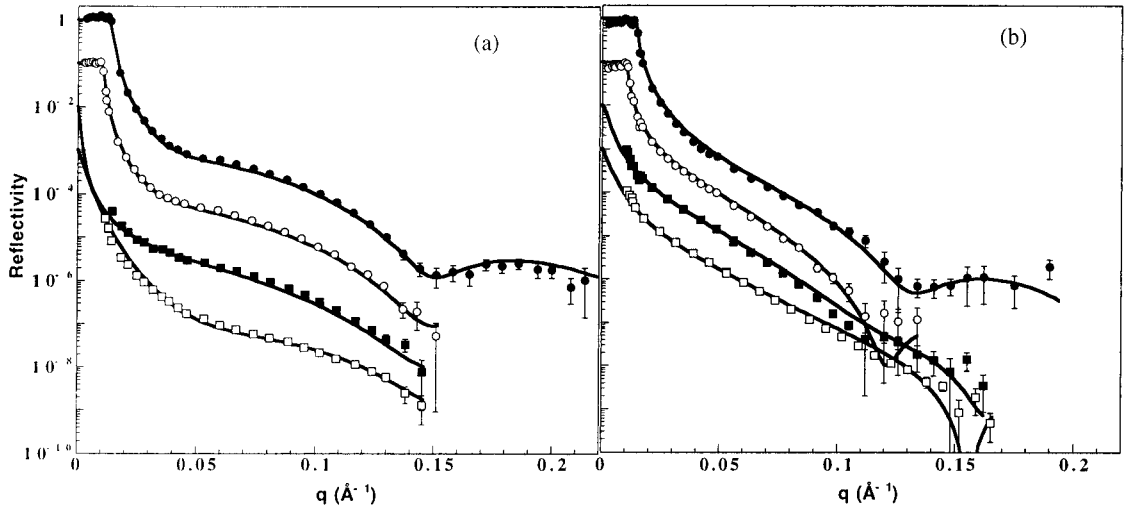

Figure 3. Neutron reflectivity profiles and fitted curves for the system (a) Si/SiO_2/h-PCPS/water and (b) Si/SiO_2/d-PCPS/water in (●) D_2O, (○) 4.5MW, (■) SMW and (□) H_2O. For clarity reflectivity from 4.5MW is divided by 10, from SMW by 100, and from H_2O by 1000. For the parameters used to fit the layers see Table 3.

value of 7.66×10^{-6} Å^{-2}. This discrepancy will be dealt with in the Discussion Section. In all cases the headgroups have a thickness of 9 ± 1 Å while the chain region is 36 $\pm$ 1 Å thick. The overall thickness of the bilayer is 54 $\pm$ 1 Å (error is smaller than the sum of each box error).

A roughness of 5 ± 1 or 6 ± 1 Å is found at the interface of each layer and is the same as for the bare blocks. About 20 ± 5% of water is detected in inner layer and of 35 to 40 ± 10% in the outer layers.

A schematic of the bilayer is shown in Figure 4.

Effect of the Peptide. The effect of the presence of *p-Antp*$_{43-58}$ in the bilayer was studied by measuring the neutron reflectivity curves of the fully hydrogenated and fully deuterated bilayers deposited on the solid surfaces from monolayers containing the peptide. The measured contrasts are given in Table 2. Figures 5 and 6 show the measured curves and the curves from the fitted model summarized in Table 4.

Data analysis was more difficult to handle than for the pure lipid systems because of the uncertainty in the determination of the theoretical peptide SLD in different contrasts. The first approach was to look for a model where the SLD varied for each contrast according with a complete substitution of the hydrogens with deuterium in D_2O and a substitution proportional to the quantity of D_2O present in SMW and 4MW. No model coherent with all data was found in this way. When up to 50% of the labile hydrogens were replaced by deuterium, the model parameters fell into the error bars of the model found with 0% substitution. It was finally decided that, with no other means of determining the exact number of protons exchanged, data would be fitted for each set of contrasts on a given sample with a unique SLD value and neglecting the effects of hydrogen/deuterium exchange on the peptide. This will be taken into account in the resulting interpretation.

Data from systems containing DPPC+*p-Antp*$_{43-58}$ were fitted by a five box model, the first box describing the oxide layer and the second the thin layer of water, whose thickness does not vary in the presence of peptide. The following three boxes describe the mixture of phospholipids and peptide. The thickness and roughness of the bilayer seem to have undergone minor changes upon the introduction of peptide while the composition is different with respect to the case of the pure phospholipid bilayer. In fact, the SLDs of the inner boxes change from -0.41×10^{-6} Å^{-2} to $(0.2 \pm 0.1) \times 10^{-6}$ Å^{-2} with hydrogenated lipids and from (7.2 ± 0.2) to $(5.2 \pm 0.1) \times 10^{-6}$ Å^{-2} with deuterated ones (cfr. Table 4). As for the outer boxes the

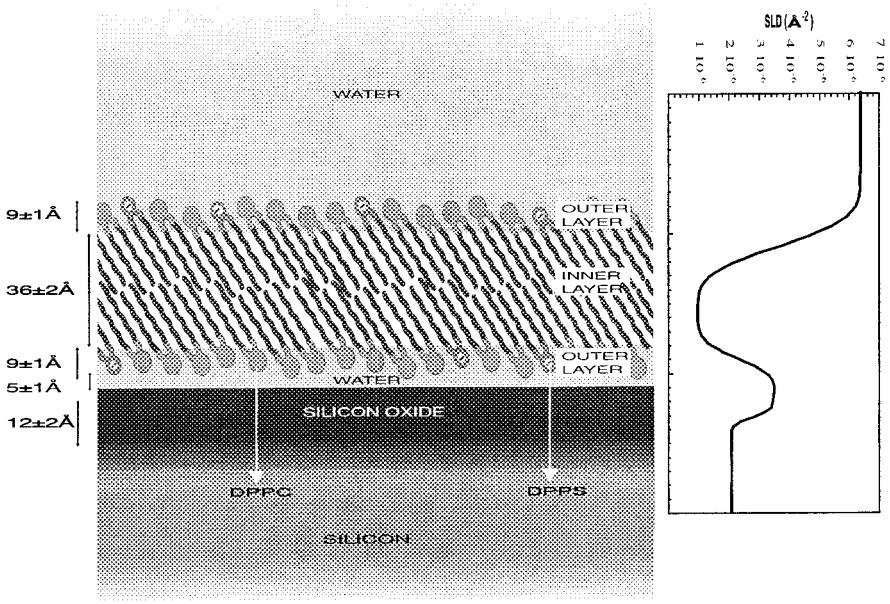

Figure 4. Schematic of the PCPS bilayer. Tilt angle of 39° accounts for the smaller thickness of the bilayer when compared with the length of two fully extented phospholipid molecules, see text. On the right, scattering length density profile for h-PCPS in D_2O (see Table 3).

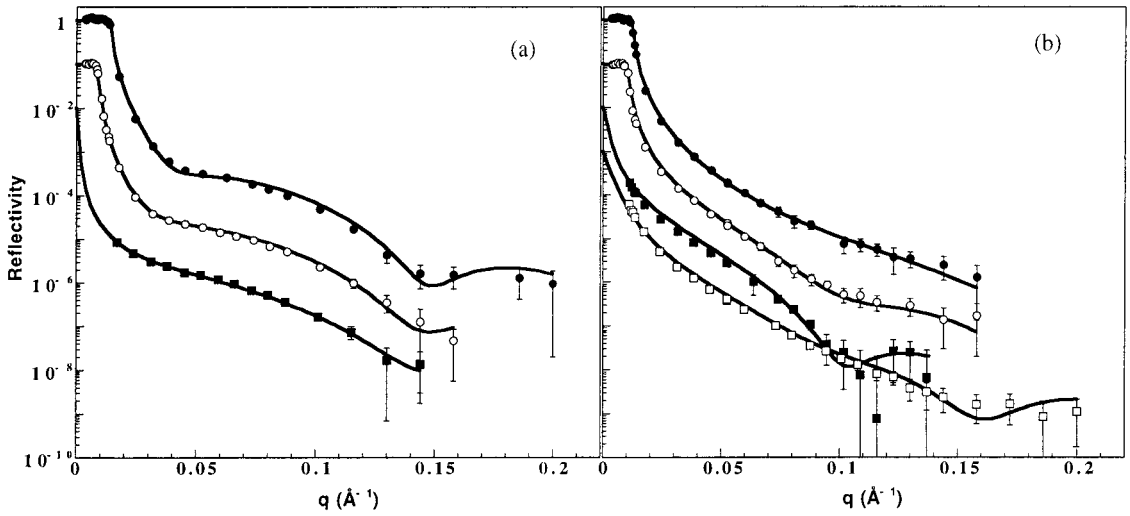

Figure 5. Neutron reflectivity profiles and fitted curves for the system (a) Si/SiO_2/h-DPPC+*p*-Ant_{43-58} /water (PBS solution) in (●) D_2O, (○) 4MW and (■) SMW and (b) Si/SiO_2/ d_{75}-DPPC+*p*-Ant_{43-58} /water (PBS solution) in (●) 5.3MW, (○) 4.5MW, (■) SMW and (□) H_2O. For clarity reflectivity from 4MW and 4.5MW is divided by 10, from SMW by 100, and from H_2O by 1000. For the parameters used to fit the layers see Table 4.

SLDs change from 1.74×10^{-6} Å^{-2} to $(1.7 \pm 0.5) \times 10^{-6}$ Å^{-2} and from 5.70×10^{-6} Å^{-2} to $(5.4 \pm 0.5) \times 10^{-6}$ Å^{-2} in the hydrogenated and deuterated cases, respectively.

Data from systems containing PCPS+*p*-$Antp_{43-58}$ are also fitted by a five box model with the first box describing the silicon oxide, the second the water layer, and the following three boxes the embedded membrane. In this case the structure of the bilayer changes with respect to the system without peptide. The outer box SLDs change from 1.83×10^{-6} Å^{-2} to $(1.7 \pm 0.4) \times 10^{-6}$ Å^{-2} and from 5.40×10^{-6} Å^{-2} to $(4.6 \pm 0.4) \times 10^{-6}$ Å^{-2} in the hydrogenated and deuterated lipids, respectively. The composition of the inner boxes also changes with the SLD going from -0.41×10^{-6} Å^{-2} to $(-0.2 \pm 0.2) \times 10^{-6}$ Å^{-2} with hydrogenated lipids and from (7.2 ± 0.2) to $(5.9 \pm 0.3) \times 10^{-6}$ Å^{-2} with deuterated ones (cfr. Table 4). The box thicknesses vary (outer boxes increase from 9 ± 1 to 13 ± 2 Å and the inner box thickness decreases from 36 ± 1 to 29 ± 2 Å) and the roughness also varies (increase from 6 ± 1 to 13 ± 2 Å).

The variations in the SLDs of the boxes with respect to the model found for the pure bilayers are consistent with the presence of peptide. In the DPPC case, eq 2 has been applied simultaneously to all data from the peptide containing samples (data from 6 and 7 in Table 2), with $x = 0$ for the inner box, SLD_{chains} taken from Table 1, SLD_{box} taken from Table 4 (bilayer + peptide). By assuming that $SLD_{peptide}$ is that given in Table 1 it is found that chains occupy 64% of the covered surface and the peptide the remaining 36%. It is more complicated to apply eq 2 to the PCPS case because of the bigger modifications to the structure (which could imply some intermixing of headgroups and chains in the various boxes and a variation in the density of the chains in the inner boxes). In fact, the data are not consistent with a simple incorporation of peptide. Only a qualitative conclusion may be drawn.

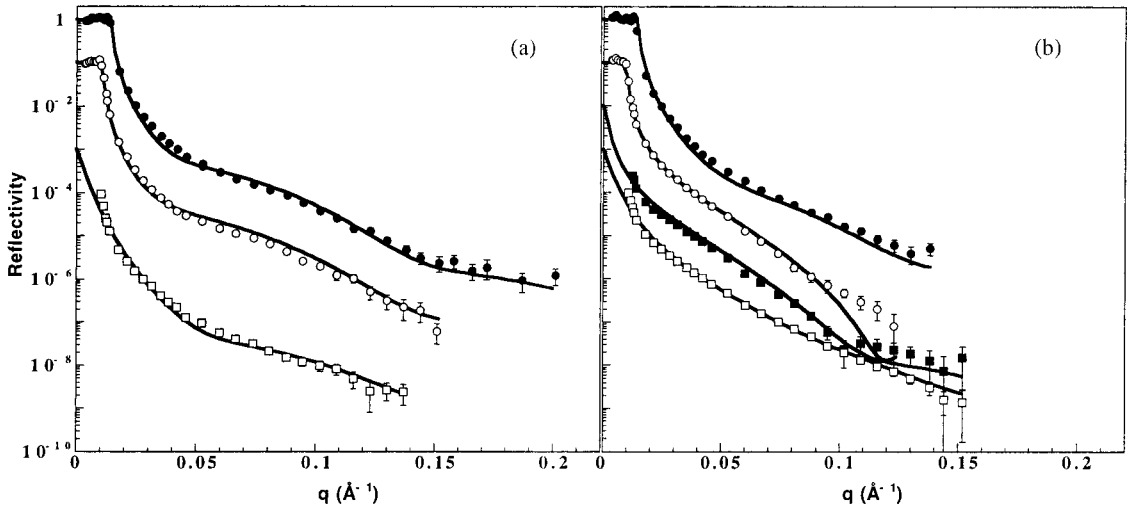

Figure 6. Neutron Reflectivity profiles and fitted curves for the system (a) Si/SiO_2/h-PCPS+*p-Ant*$_{43-58}$ /water (PBS solution) in (●) D_2O, (○) 4.5MW and (□) H_2O and (b) Si/SiO_2/d-PCPS+*p-Ant*$_{43-58}$ /water (PBS solution) in (●) D_2O, (○) 4MW, (■) SMW, and (□) H_2O. For clarity reflectivity from 4.5MW and 4MW are divided by 10, from SMW by 100, and from H_2O by 1000. For the parameters used to fit the layers see Table 4.

A 5 to 10% increase in the percentage of water is found at the interface in both cases.

Discussion and Conclusion

In this paper we have presented the results of a neutron reflectivity study of the structure of deposited phospholipid bilayers and its modification in the presence of the peptide *p-Antp*$_{43-58}$. The effect of the introduction of negative charges in the lipid headgroups on the adsorption of peptide was investigated.

The oxide layer on the silicon crystals was characterized first. The importance of a good characterization of such a layer has already been discussed elsewhere.[18] In most of the published reflectivity results on silicon crystals (see ref 14 and references therein) where the surface had received a chemical treatment (HF and/or peroxide treatments), water penetration in the oxide has always been detected. Here, a better oxidation[20] and probably a better polished surface has led to a more compact layer.

The thickness of the water film at equilibrium between the substrate and lipid heads was also accurately determined. It is reproducibly around 5 Å, and it is consistent with the equilibrium value.[25] The presence of 10% mol/mol charged heads or of ions in solution does not seem to influence that value. This is surprising and further studies are being carried out to investigate this aspect in more detail.

The structure, thickness, roughness, and coverage rate of the bilayer was finely determined. The position of headgroups and chains was determined at 1 Å precision. Our results agree with the literature data on phospholipid bilayers and stacked bilayers[26−30] but greater details are given on the structure and composition. The chain thickness (36 ± 1 Å) is smaller than the length of two fully extended lipid chains (41 Å, see Table 1). This means that chains either intermix or, more likely, they are tilted 39° from the normal. The percentage of water in the bilayer necessary to fit the data is interpreted as follows. The bilayer covers about 80% of the available surface which explains the amount of water found in the hydrophobic inner box. This percentage agrees with that found by AFM and transfer rate measurements on similar samples.[16] Recent AFM studies of Langmuir−Blodgett bilayers deposited on mica substrates[31] suggest that defects are micrometer size patches of bare substrate in an otherwise complete bilayer. Here, the neutron beam transverse (spatial) coherence length is 0.1 μm and the coherence length projected on the sample is $0.1\ \mu m/\theta$, that is of the order of tens of microns. Neutron specular reflection gives no information about the in-plane structure at the interface and therefore it is not possible to determine whether the water is uniformly distributed among the lipids or if it concentrates in holes of the bilayer. The higher percentage of water in the outer boxes is consistent with the fact that headgroups are hydrophilic and surrounded by water molecules. The loosely bound molecules above the headgroups cannot be distinguished from the bulk water in our experiments while those between the heads can be seen. If we assume that two water molecules are tightly bound to the headgroups, as suggested by ref 32 these will occupy about 15% of the headgroup volume which is consistent with our results.

The discrepancy between the fitted and theoretical SLD of the deuterated chains could be explained by allowing a small percentage of headgroups in the chain region (possibly at the boundary of the domains), or that the volume occupied on the surface is about 6% bigger than that given in Table 1 (indicating a more disordered structure at the interface than that predicted for the gel phase in solution) or more likely a combination of the two.

After introduction of *p-Antp*$_{43-58}$, the bilayer structure becomes more complicated. There are more unknown parameters; the uncertainty in the thicknesses increases but is not larger than 2 Å. In the presence of zwitterionic lipids (DPPC), the structure of the bilayer is maintained, in terms of thickness and roughness, while changes in the scattering length density are consistent with an incor-

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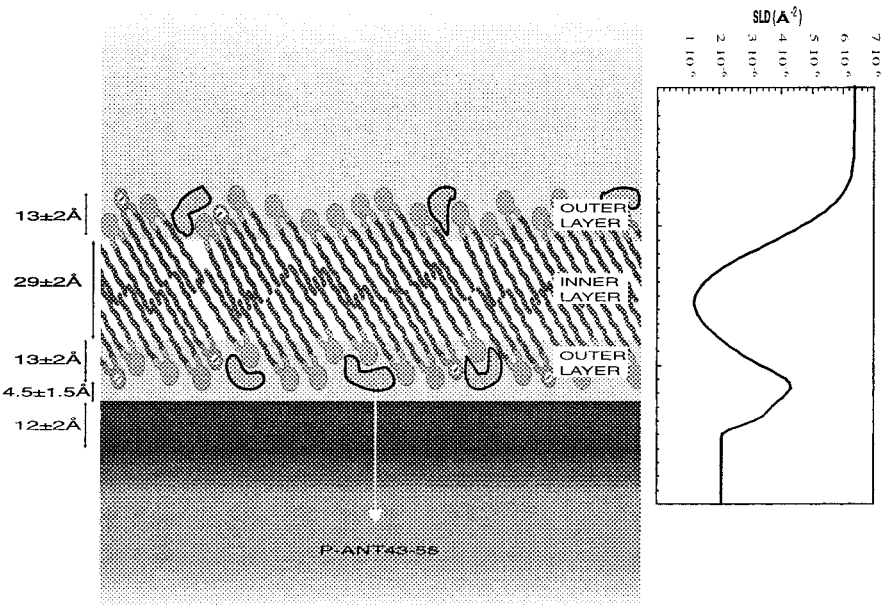

Figure 7. Schematic of the PCPS bilayer with the embedded *p-Ant*$_{43-58}$. Chains are tilted for the same reason as Figure 4. On the right, scattering length density profile for h-PCPS + peptide in D_2O (see Table 4).

poration of peptide either segregated in holes of the bilayer or uniformely distributed in the membrane. Since incorporation inside the membrane is likely to perturb its thickness and/or roughness, we suggest that the peptide is segregated in the holes. While the thickness of the bilayer is well defined by the curves measured from h-DPPC, the curves measured from d_{75}-DPPC better constrain the composition of the layers.

When peptide is added in the presence of 10% mol/mol negatively charged lipids (DPPS), the structure is modified. The most remarkable effect of the presence of peptide in the membrane is the significant increase of the roughness from 6 ± 1 Å, in the bare bilayer (which is roughly the same as the substrate), to 13 ± 2 Å in the bilayer plus peptide. A quantification of the peptide present is difficult but the model parameters given in Table 4 suggest that it is mainly segregated into the outer boxes (Figure 7). The deuterated peptide would be more helpful for these determinations since it is found that the protonated one has SLD similar to that of the protonated headgroups, but the compound is not yet available. A more precise indication of the position of the peptide should also be possible through fluctuation correlations in the plane determined by off-specular reflectivity (work in progress).

A 5 to 10% increase in the percentage of water is found at the interface in both cases, which may either mean that less lipids have been deposited than in the absence of peptide or that it is the peptide hydration water.

The above results suggest that even before the deposition on the solid substrates there is a difference in the adsorption behavior of the peptide when the monolayer is formed by pure DPPC or DPPC + 10% mol/mol DPPS. At the air/water interface, when the monolayer is formed by uncharged lipids the peptide is mainly located at the water surface between the lipid molecules; in the presence of negative charges in the headgroup region the peptide also adsorbs under the monolayer:[15] it is likely that when it is transferred to the solid substrate it remains embedded in the headgroups.

Finally, neutron reflectivity is a suitable technique for studying the interaction of small molecules, like peptides, with phospholipid single bilayers. The effect of negative charges on the interaction was visible. The deposited bilayer, with and without peptide, was found to be stable in time and the deposition technique very reproducible. This has opened the way to further studies, currently in progress,[33] toward more fluid systems and to off-specular measurements for a better study of the in-plane structure and fluctuations.

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