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Phospholipid bilayer formation at a bare Si surface: a time-resolved neutron reflectivity study

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Abstract

Neutron reflectivity was applied to monitor *in situ* the adsorption of small unilamellar phospholipid vesicles on a solid bare hydrophilic Si interface. The obtained reflectivity curves are consistent with the rupture and fusion model for the adsorption of phosphatidylcholine vesicles to solid interfaces. The results show details of the adsorbed bilayer system at ångström resolution and indicate the presence of a thin ∼6 Å thick water leaflet that separates the bilayer from the Si surface. The resolved structural details provide the basis for further investigation of processes such as adsorption and penetration of peptides and proteins towards the supported bilayer at high resolution.

1. Introduction

The design and structure of biomimetic films along a planar surface is of great interest for the development of biocompatible interfaces, the construction of biosensors based on membrane receptors, and as biophysical model systems for studying the interaction of biomolecules with membrane surfaces (e.g. [1]). A basic step in the design of such systems is the preparation of phospholipid molecules in the form of planar bilayers along adequate interfaces. The deposition of lipid layers onto supports has been done so far by using two preparation approaches: (i) Langmuir–Blodgett transfer techniques [2, 3], or (ii) spontaneous fusion of small uni-lamellar vesicles onto solid–liquid interfaces [4–6].

In spontaneous fusion of unilamellar vesicles, the self-assembly of a single phospholipid bilayer is easily obtained on charged and hydrophilic solid interfaces [4–6].

By Langmuir–Blodgett transfer techniques multi-layered structures are obtained in a defined sequence [2, 3, 7]. Less defined multi-layered structures are found by the self-assembly

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of multi-lamellar vesicles on solid–liquid interfaces [8] or at the air–water interface [9] depending on the vesicle concentrations used.

The underlying self-assembly and fusion processes involved in the creation of single phospholipid bilayers on hydrophilic planar surfaces by spontaneous fusion have attracted the interest of many researchers and various studies using microscopic and spectroscopic methods including reflection interference contrast microscopy [10], total internal reflection fluorescence microscopy [11, 12], atomic force microscopy (AFM) [13], surface plasmon resonance [14], impedance changes [15], Fourier transform infrared spectroscopy [16, 17] and quartz crystal microbalance (QCM) measurements [18]. The results of these studies have shown that bilayer covered supports were produced; the stability, homogeneity and the general process of the adsorption of phospholipid vesicles towards planar hydrophilic solid surfaces were also described.

There is, however, still a need to resolve the fine details of the structures obtained, an issue that is most desirable in order to elucidate the position of molecular subunits along the bilayer membrane or of embedded molecules in mixed systems with lipids, peptides or proteins. This request cannot be fully covered if only the above methods are applied, so x-ray and neutron reflectivity and grazing incidence diffraction studies are needed. Yet it is difficult to obtain well defined structures only by x-ray reflectivity measurements due to the low electron density contrast of phospholipid bilayers and water, and the low brilliance of currently available neutron sources complicates the acquisition of well resolved neutron reflectivity curves beyond a sufficiently high q-range. Therefore, up to now these techniques have been exploited only in a few studies for the investigation of the structure of supported phosphilipid bilayers in detail, and for the layer formation process itself. Johnson *et al* [5] measured the thickness and uniformity after the adsorption of a single phospholipid bilayer onto a planar quartz surface with specular neutron reflectivity. Koenig *et al* [19] studied the adsorption of phospholipids towards bare silicon surfaces in another study using neutron reflectometry. They showed the immediate adsorption of a self-assembled single phospholipid bilayer within a few minutes of introduction of a vesicle solution into the sample cell; this was in agreement with results obtained by quartz crystal microbalance investigations [18]. The interaction of a helical peptide fragment with a supported phospholipid bilayer was investigated by neutron reflectivity only recently [20]. The distribution and insertion of the peptide into the headgroup regions of the phospholipid bilayer could be determined with a resolution in the 1 Å range.

We have used the technique of neutron reflectivity to complement and extend the investigations mentioned above on the structure and process of formation of single phospholipid bilayers as they associate along hydrophilic solid–liquid interfaces, and to probe the feasibility of time-resolved neutron reflectometry for studies that examine the spontaneous adsorption of single phospholipid bilayers from solutions of uni-lamellar vesicles *in situ*. Choosing time steps between 2 and 10 min for data integration, the formation process was monitored over the accessible q-range ($q < 0.4$ Å^{-1}) at the time-of-flight neutron reflectometer D17 at ILL, Grenoble. The results obtained on the monitored adsorption process are fully compatible with the rupture and fusion process observed with AFM [13] and QCM [18], giving evidence of an incomplete coverage at the Si interface within the first minutes after introduction of the vesicle solution. In a certain time range, a uniform layer is built up. The final phospholipid bilayer exhibits no barely recognizable defects from the reflectivity measurements. This is obtained several tens of minutes after vesicle exposure. The structural model that fits the collected data depicts a single bilayer with a distinct independent water layer present between the solid interface and the phospholipid membrane.

2. Experiments and methods

Neutron reflectivity measurements were made at the time-of-flight neutron reflectometer D17 at ILL, Grenoble, France. Measurements were performed in the q-range of $0.01 < q < 0.085$ Å^{-1} with 2 min of acquisition time, and between $0.044 < q < 0.42$ Å^{-1} within 10 min. This corresponds to a wavelength band of $1.3 < \lambda < 10.9$ Å at $\theta = 0.5°$ and 2.2°, respectively.

The beam of rectangular cross-section 0.5×20 mm^2 impinged on the samples at the solid–liquid interface through the silicon block [21]. The scattered neutrons were recorded with a 2D ^{3}He-detector system.

A home-built sample cell for neutron reflectivity studies of solid–liquid interfaces was used [22]. It consists of a PTFE trough of inner dimensions $72 \times 42 \times 3$ mm^3, corresponding to a sample volume of ∼9 ml, that was sealed with a Viton O-ring against the silicon block. Fluids could be added through stainless steel inlet and outlet tubes that are mounted in opposite corners of the trough. The trough–silicon sandwich was clamped between two thermostatted aluminium supports allowing for temperature adjustment within ±0.5 K.

The Teflon trough and the silicon surface were cleaned prior to the incorporation into the sample cell by sonication in aqueous HELMANEX solution and by the standard RCA method, respectively. The cleaned silicon surface was covered with a native silicon oxide layer.

Dimyristoylphosphatidylcholine (DMPC) was used as purchased (Avanti Polar Lipids, Alabama). The lipid was suspended in D_2O (1 mg ml^{-1}) and extruded to small unilamellar vesicles (SUVs) of various vesicle diameters (50 and 5000 nm) [13, 23]. The vesicle suspensions during preparation and measurement were kept at 30 °C, which is above the main phase transition of 24.1 °C for DMPC. After measurement of the signal of the bare silicon interface as reference, the vesicle solution was directly injected into the sample cell and the deposition studies started immediately.

The specular reflectivity curves were analysed by applying a standard fitting routine [24] based on Parratt's dynamical approach on the reflectivity of stratified media (see [25, 26] and references therein). The interface is therein modelled consisting of slabs of uniform neutron scattering length density, $\rho^s = Nb$, where N is the number density in units of 1 Å^{-3} and b the scattering length in units of Å. From the slab model fits to the experimental data the thickness, d_L, and the scattering length density, ρ_L^s, of each layer were extracted. The scattering length density for Si was fixed at 2.073×10^{-6} Å^{-2} and for D_2O at 6.366×10^{-6} Å^{-2}, taking the theoretical values. No roughness estimate was included in the fitting procedure.

3. Results

Specular neutron reflectivity patterns collected in the q-range $0.01 < q < 0.4$ Å^{-1} showed the evolution of a DMPC structure adsorbed to the bare silicon interface within about 30 min after injection of the freshly prepared SUV solution into the sample cell (figure 1). The increase in reflected intensity at $q = 0.09$ Å^{-1} reached saturation within less than one hour (figure 1, inset) in agreement with previous results given by Koenig *et al* [19], who used sonicated DPPC vesicle solutions.

Modelling of the reflectivity patterns by slabs of uniform scattering length densities from the system in the final state showed the structure of a single homogeneous DMPC layer of ∼46 Å (figure 2, table 1). The layer is separated from the silicon interface by a small layer of ∼6 Å thickness with a neutron scattering length density of pure D_2O.

The DMPC structure could be fitted either with a single slab of neutron scattering length density (straight curves in figure 2) or with a three-slab model resembling the hydrophilic headgroup–hydrophobic alkyl chain–hydrophilic headgroup structure of a single phospholipid bilayer (dotted curves in figure 2).

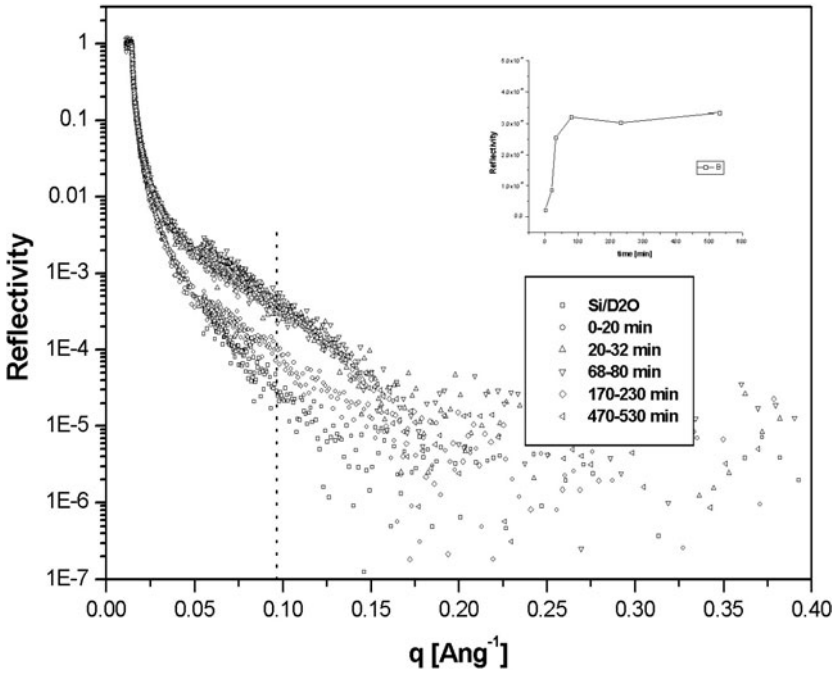

Figure 1. Neutron reflectivity curves obtained during adsorption of DMPC vesicles at the Si surface in D_2O. The inset shows the change in reflected intensity at fixed $q = 0.09$ Å^{-1}.

Table 1. Parameters derived from slab model fits to the reflectivity curves (figure 2).

	DMPC					
	38 min		68 min		148 min	
	d (Å)	ρ^s (10^{-6} Å^{-2})	d (Å)	ρ^s (10^{-6} Å^{-2})	d (Å)	ρ^s (10^{-6} Å^{-2})
			MODEL 1			
Si	—	2.073	—	2.073	—	2.073
D_2O	6.2	6.366	6.84	6.366	6.2	6.366
DMPC layer	37.0	1.053	35.88	−0.202	37.0	−0.345
D_2O	—	6.366	—	6.366	—	6.366
χ^2	7.65		0.678		2.99	
			MODEL 2			
Si	—	2.073	—	2.073	—	2.073
D_2O	5.8	6.366	5.8	6.366	5.8	6.366
Headgroup layer 1	10.2	2.501	10.2	2.501	14.2	2.501
Hydrocarbon layer	24.7	0.937	24.7	−0.337	24.4	−0.337
Headgroup layer 2	8.9	2.885	8.9	2.885	7.5	2.761
D_2O	—	6.366	—	6.366	—	6.366
χ^2	4.58		0.246		1.15	

Table 1 shows two different fit models for the structure that were tested and found to be reasonably good. The only difference is the entering of lipid molecules as uniform components

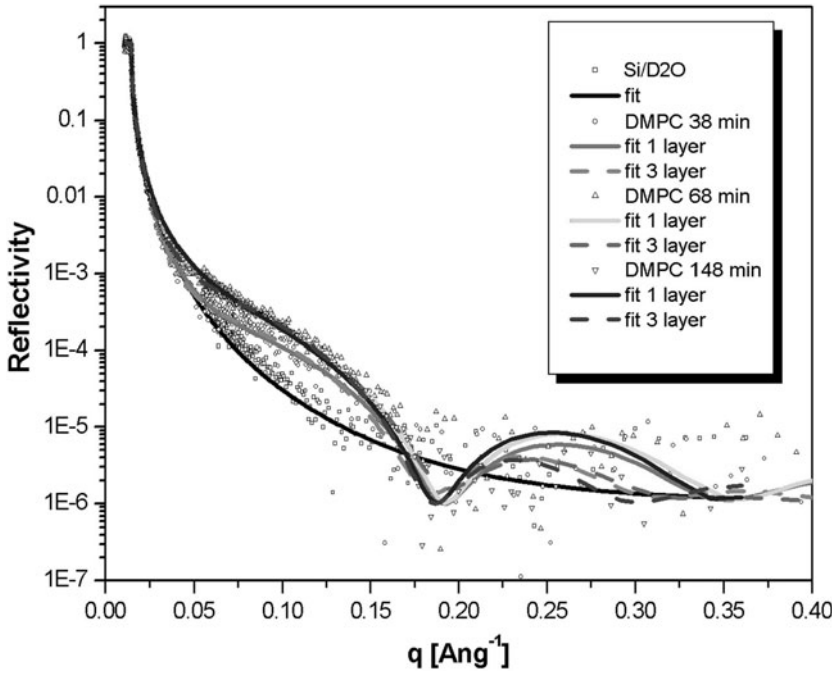

Figure 2. Calculated slab model fits to reflectivity curves measured at different times during the adsorption of DMPC vesicles to the Si surface in D_2O. Fit parameters are given in table 1.

(MODEL 1) or in separate units, representing the headgroups and chain regions (MODEL 2). The three-slab model showed slightly improved χ^2 values as compared to the one-slab model (table 1). At increased measurement times, i.e. improved signal statistics and better signal-to-noise ratio, the fits obtained by the more detailed three-slab model of the DMPC bilayer structure were clearly a better approach to the reflectivity patterns. The scattering length density in the hydrophobic region of the layer structure changed from slightly positive values indicating the presence of holes filled with D_2O within the adsorbed bilayer structure to a negative scattering length density as calculated for a homogenous layer of pure hydrocarbon chains (table 1). The final neutron scattering length densities obtained in the model fits showed the DMPC headgroups fully hydrated with 12–14 water molecules per phospholipid in accordance with our previous findings [27] and with results from x-ray diffraction measurements [28, 29].

While the reflectivity patterns were still evolving, the collected data could be analysed in terms of an incompletely formed adsorption layer. This can be done either by superposition of the reflectivity pattern of the bare silicon interface and a complete phospholipid layer structure with holes or by a layer structure with a neutron scattering length density in between the density of D_2O and pure DMPC. These approaches represent two different cases of incomplete coverage of an adsorbed layer to an interface [30].

In the case of superposition of the reflectivity pattern by the reflectivity given by the bare silicon interface and the complete phospholipid layer structure it would indicate the evolution of independent areas of phospholipid covered and uncovered regions at the silicon interface with dimensions greater than the coherence length of the neutron beam (figure 3(a)). In the case of a layer structure with diminished neutron scattering length density as compared to D_2O the extensions of the interfacial regions covered or uncovered with phospholipid layer, respectively, have to be below this coherence length (figure 3(b)).

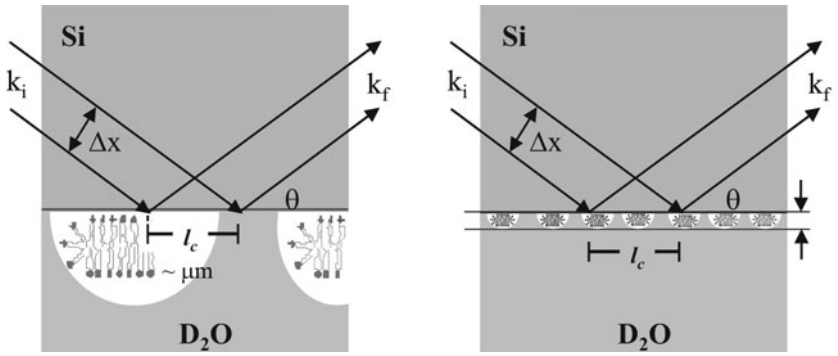

Figure 3. Schematic description of reflected neutron beam at the solid–liquid interface in the case of volumes with dimensions greater (left) and smaller (right) than the coherence length l_c of the neutron beam.

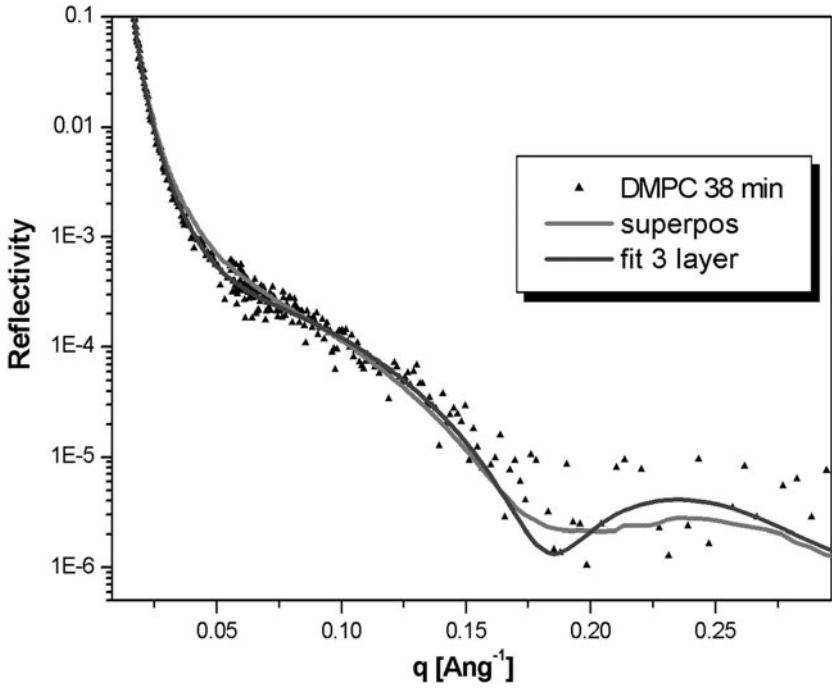

Figure 4. Calculated slab model fits to the reflectivity curves measured of the adsorption of DMPC vesicles to a Si/D_2O interface after 38 min. The reflectivity curve is equally well described by a model assuming a single layer of reduced scattering length density as by a 50:50 superposition of two reflectivity curves for the bare Si interface and an interface covered completely by a homogeneous DMPC bilayer with a total thickness of 43.8 Å as given in table 1, MODEL 2.

The obtained reflectivity pattern recorded 38 min after injection of the DMPC sample suspension can be fitted with both approaches almost equally well (figure 4). Thus at the dimension of the coherence length of the neutron beam the build-up of the adsorbed phospholipid bilayer at the silicon interface is not resolvable in terms of a subsequent rupture of SUV at the interface and following fusion as shown in figure 5 and suggested on the basis

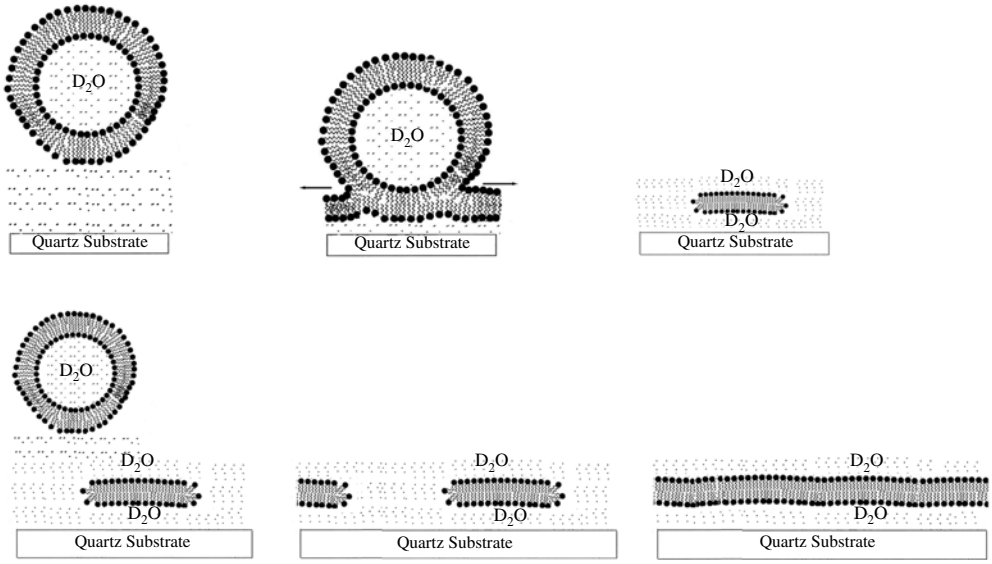

Figure 5. Schematic description of the adsorption of uni-lamellar phospholipid vesicles towards a solid hydrophilic interface.

of recent results obtained by AFM [13] and fluorescence microscopy investigations [12] or as discussed for the results of quartz microbalance investigations [18, 31, 32].

The evolution time span prior to the complete coverage of the Si interface when no further change in the reflectivity curve takes place is significantly larger as compared to the time reported in comparable QCM studies. This may be due to the large sample area used compared to the small area in the QCM cell. More likely is the explanation that the reflectivity curves probe the extent of layer defects in the adsorbed phospholipid layer that slowly decrease with time. The fusion of single-bilayer discs has been shown to be much slower than the initial fusion and rupture of intact vesicles [13].

The initial process of individual rupture and adsorption of single DMPC SUVs on the bare silicon surface happens in time ranges which are too short to be monitored by current time-of-flight neutron reflectivity experiments with useful statistical meaning. Differences in the adsorption and formation of the phospholipid bilayer due to different vesicle sizes (50 and 500 nm diameter) were not observed. Nevertheless, the recorded reflectivity patterns give a lower limit in the statistical relevance for the measurement of single phospholipid bilayers adsorbed to hydrophilic interfaces in aqueous environment. They also underpin previous investigations that this adsorption process in this time range is not affected by vesicle size or temperature for the phospholipids in the gel state [31, 33, 34].

Heating the sample up to 70 °C did not affect the adsorption of the single DMPC bilayer towards the silicon interface. At this temperature a slight thinning of the membrane thickness and a slow decrease in bilayer neutron scattering length density occurred (data not shown). This might indicate a slow evolution of defects and pores in the adsorbed layer as a beginning of dissolution of the single phospholipid bilayers and transformation back to SUVs.

4. Conclusions

By means of neutron reflectivity the spontaneous formation of a single phospholipid bilayer adsorbed to a bare hydrophilic Si surface was monitored *in situ*. The process monitored

is described as a rupture and fusion mechanism with immediate rupture and adsorption of individual phospoholipid vesicles at the bare Si covering most of the surface within a few minutes after exposure of the interface to a suspension of small unilamellar vesicles. This initial process is too fast to be monitored with neutron reflectivity. In a second stage the growth of the bilayer covered area by fusion of single spots is observed until finally a homogenous stable and fluid phospholipid bilayer covers the interface. The bilayer is separated from the Si surface by a thin water leaflet so that both leaflets of the adsorbed phospholipid membrane are fully hydrated. The obtained neutron reflectivity curves clearly indicate detailed structural features of the adsorbed bilayer using simple slab model fittings. These detailed features can be used for the structural analysis of processes such as adsorption and penetration of peptides and proteins into the supported bilayer or the distribution of surfactants or DNA into such systems.

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