

## Surface-sensitive X-ray and neutron scattering characterization of planar lipid model membranes and lipid/peptide interactions

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### **KEYWORDS:**

lipid membrane models, protein crystallography, thermally disordered membrane structure, protein folding, Langmuir monolayers, self-assembled monolayers, hybrid bilayer models, polymer-supported tethered lipid bilayers

### **ABSTRACT**

Recent progress in submolecular level structural investigations of planar lipid model membranes using scattering techniques is reviewed. Particular emphasis is placed on the quantification of peptide/lipid and protein/lipid interactions. Floating phospholipid monolayers on aqueous sub-phases ("Langmuir monolayers") enable full physicochemical control of a membrane mimic, thus providing unique opportunities for investigations of the interaction of peptides with biomembrane surfaces. Particular progress has been recently made with surface-sensitive diffraction methods to characterize molecularly thin protein crystal sheets. We proceed to describe (multi-) bilayer systems for which the distribution of submolecular fragments has been determined providing a fully resolved picture of their thermally disordered structure using the 'liquid-crystallography' approach of White and coworkers. This approach has recently been extended to tackle basic problems of membrane protein folding. Applied to solid-state interfaces with a fluid or gas phase, scattering methods provide invaluable tools for the Ångstrom-scale characterization of systems such diverse as uniaxially oriented proteins in molecular layers attached to self-assembled monolayers (SAM's), hybrid bilayer membranes (HBM's), or polymer-cushioned, tethered bilayer membranes.

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## GLOSSARY OF ABBREVIATIONS

### phospholipids:

PA	–	phosphatidic acid
PC	–	phosphatidylcholine
PE	–	phosphatidylethanolamine
PG	–	phosphatidylglycerol
PS	–	phosphatidylserine
DP	–	dipalmitoyl (di-C16:0)
DM	–	dimyristoyl (di-C14:0)
DL	–	dilauroyl (di-C12:0)
DO	–	dioleoyl (di-C18:1)

### phospholipid monolayer phases:

LE	–	liquid expanded
LC	–	liquid condensed
SC	–	solid condensed

### peptide and protein systems:

SP-B/C	–	pulmonary surfactant peptide B/C
PLA <sub>2</sub>	–	phospholipase A <sub>2</sub>
GOx	–	glucose oxidase
Vpu	–	HIV-1 virus protein U
bR	–	bacteriorhodopsin
PM	–	purple membrane
BSA/HSA	–	bovine/human serum albumin
AA	–	amino acid

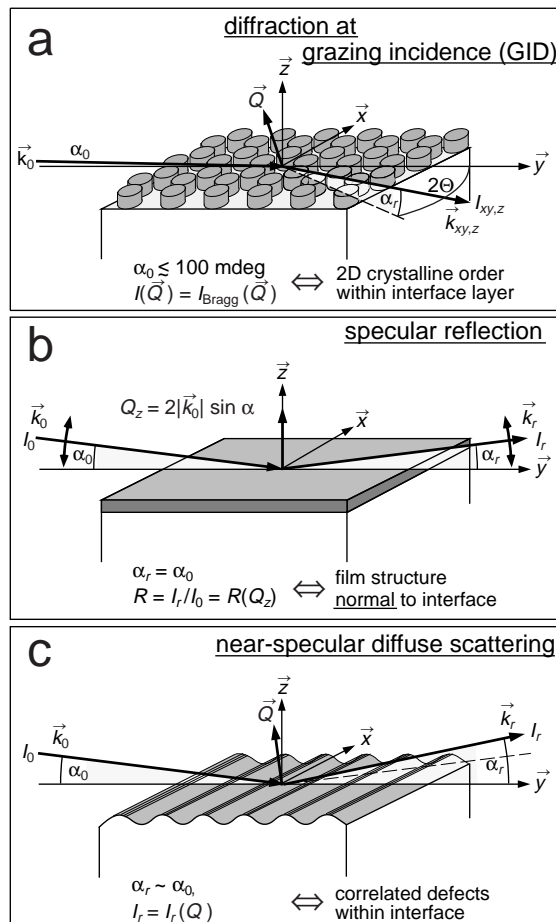
### other:

SAM	–	self-assembled monolayer
HBM	–	hybrid bilayer membrane
BLM	–	bilayer lipid membrane
NBF	–	Newton black films
2D/3D	–	two-/three-dimensional
ESRF	–	European Synchrotron Radiation Facility (Grenoble, France)
DESY	–	Deutsches Elektronensynchrotron (Hamburg, Germany)
APS	–	Advanced Photon Source (Argonne National Laboratory, IL, U.S.A.)
NIST	–	National Institute of Standard and Technology (Gaithersburg, MD, U.S.A.)
GIXD	–	grazing-incidence X-ray diffraction
XAFS	–	X-ray absorption fine structure spectroscopy
SLD	–	scattering length density
VRDF	–	volume-restricted distribution function
FWHM	–	full width at half maximum
FT-IRRAS	–	Fourier transform infrared reflection absorption spectroscopy
AFM	–	atomic force microscopy
FRAP	–	fluorescence recovery after photobleaching
MBE	–	molecular beam epitaxy
RH	–	relative humidity
PVP	–	poly(vinylpyrrolidone)

## I. INTRODUCTION

By providing detailed three-dimensional structures of proteins and nucleic acids, as well as protein/nucleic acid complexes, on the scale of individual atoms molecular biology and high-resolution x-ray crystallography have revolutionized our views on biology, medicine, disease, and morphogenesis in terms of biological supramolecular self-assembly. Molecular machines that mend the games of life have thus become comprehensible to a level of detail that has not been dreamt of a mere few decades ago. Yet, the availability of such structures, beautiful as they are, solves only one facet of the problem that involves the question, how does life work? Another key ingredient for a deeper understanding is the spatial organization of these machines within the cell, mediated largely by membrane structures, composed of molecular bilayers only 5 nm thick, which are laterally disordered in their nature and thus resist analysis with the very same methods that have been so successfully employed to explore the internal structure of proteins. As if devised to complicate their molecular-scale analysis, such lipid membranes are highly complex blends of several classes of amphiphilic building blocks that undergo continuously changes in their compositions, topology, and thus, functionality. In order to at least unveil general principles, if not the full details, of their functioning in terms of their physical and physico-chemical properties, model systems have been successfully employed to start unravel this constituent of the secrets of life.

Planar lipid model systems – such as lipid *Langmuir* monolayers (Möhwald, 1990, Möhwald *et al.*, 1995), substrate-supported lipid bilayers or multi-bilayer stacks (Safinya, 1997, Nagle and Tristram-Nagle, 2000), bilayer lipid membranes (BLM's: Diederich *et al.*, 1998, Hanyu *et al.*, 1998, Bezrukov and Winterhalter, 2000, Krylov *et al.*, 2000) and their inside-out counterparts, Newton black films (NBF's: Bélorgey and Benattar, 1991, Benattar *et al.*, 1999, Cuvilliers *et al.*, 2000a) – have in the past been frequently used as models, of different degrees of significance, for biological membranes and particularly employed to study lipid-lipid interactions and the self-



organization of lipids into biomimetic supramolecular aggregates. More recently, complex supramolecular architectures, such as polymer-cushioned or tethered lipid bilayers (Knoll *et al.*, 2000, Sackmann and Tanaka, 2000), self-assembled monolayers (SAM's), covalently linked to planar solid surfaces, (Ulman, 1991) or hybrid bilayer membranes (HBM's) composed of SAM's and transferred bilayers (Meuse *et al.*, 1998) have been employed to create stable and versatile membrane mimics. All of these systems, with the exception of BLM's that are too small in lateral extension, are very well amenable to the structural characterization by scattering methods. Thus, surface-sensitive x-ray and neutron scattering (Als-Nielsen and Möhwald, 1991, Als-Nielsen *et al.*, 1994, Blasie and Timmins, 1999, Tolan, 1999) has made important inroads into various disciplines for the characterization of surfaces and interfaces in molecular and submolecular detail. Lipid/peptide interactions determine many structural and functional aspects in modern life science. This paper summarizes

Figure 1: Surface-sensitive X-ray or neutron scattering geometries for various types of experiments.

recent developments in surface-sensitive scattering, with an emphasis on reflectivity measurements, in the structural and functional characterization of planar lipid systems, in particular in the context of investigations of lipid/peptide interactions.

*Langmuir* monolayers of amphiphilic compounds – such as fatty acids and esters or phospholipids – floating at the air/water interface are frequently investigated objects of research both in materials and life science as well as the Physics of low-dimensional systems (McConnell, 1991, Knobler and Desai, 1992, Möhwald, 1995). Among the most powerful methods to date for the investigation of their structures on a submolecular level are surface sensitive scattering techniques (Als-Nielsen and Möhwald, 1991, Als-Nielsen *et al.*, 1994, Weissbuch *et al.*, 1997): The diffraction of synchrotron X-ray radiation upon grazing incidence (GIXD), specular reflectivity measurements using X-rays or cold neutron beams, or diffuse (near-specular) scattering, *c.f.* Fig. 1.

GIXD has obtained extensive attention recently for its unique capabilities in determining the spatial organization of linear alkane compounds in partially ordered – hexatic or crystalline – surface phases (Dutta *et al.*, 1987, Kjaer *et al.*, 1987, Kaganer *et al.*, 1998, Kaganer *et al.*, 1999). It has been considerably boosted by the advent of third generation synchrotron radiation sources (Frahm *et al.*, 1995), which enable high-quality measurements within short illumination times and have also permitted diffraction measurements from more demanding systems such as molecular protein crystal sheets (Haas *et al.*, 1995, Verclas *et al.*, 1999, Weygand *et al.*, 1999, Lenne *et al.*, 2000). Generally, domain sizes are in the  $\mu\text{m}$  range, and since beam footprints on planar samples are of the order of  $\text{cm}^2$  in size, this implies that diffraction occurs from a randomly oriented in-plane crystal powder. For two-dimensional (2D) lattices of simple molecules, such as linear alkanes, the structure factors,

$$F_{hk}(Q_z) = \sum_j f_j \exp(i(Q_{hk}R + Q_z z_j)) \quad (1)$$

may be worked out in detail (Als-Nielsen and Kjaer, 1989). As the scattering centers (characterized by the atom form factors,  $f_j$ ) are essentially confined to a plane, extending along the  $\hat{x}$  and  $\hat{y}$  axes at  $z = 0$  and thus sampled by  $R = (x, y)$ , the diffracted intensity  $I_{hk}(Q_{hk})$  is spread out in 1D Bragg rods along  $Q_z$  where  $\vec{Q}$  is momentum transfer and  $h$  and  $k$  are the Miller indices. Thus,

$$I_{hk}(Q_{hk}) = |V(Q_z)F_{hk}(Q_z)|^2 \exp(-Q_z^2 \sigma^2) \quad (2),$$

where  $V$  accounts for the interference between waves propagating upward and down within the surface film upon multiple scattering events (Vineyard, 1982) and  $\sigma$  is a measure of the surface roughness (see below). The coherence of the lattice, characterized by the lattice constants  $d_{hk} = 2\pi/Q_{hk}$ , is given via the Scherrer equation (Guinier, 1968) by

$$L \approx 0.9 \frac{\lambda}{\Delta \cos \theta_{hk}} \quad (3)$$

in which  $2\theta_{hk}$  is the horizontal scattering angle and  $\Delta$  is the resolution-corrected FWHM line-width of the Bragg rod.

Specular reflectivity measurements in turn reveal information on the electron density distribution along the surface normal. Moreover, utilizing the knowledge of the chemical structure of the film-forming compounds, one may extract information on the three-dimensional organization in a quite general approach, composition-space refinement (Vaknin *et al.*, 1991, Wiener and White, 1991b). An overview of a basic quantitative assessment is given below. In distinction from diffraction, reflectivity measurements are not limited to highly ordered systems but do provide information on molecular subfragments which do not participate in the ordering within a hexatic or crys-

talline phase – such as the lipid headgroups. Thus, reflection measurements have great potential for probing the static structure of particularly the headgroups in lipid surface monolayers on the sub-molecular level. This is particularly valuable for investigations of lipid surfaces in a biophysical context: Biomembranes are disordered systems and many physiologically important interactions take place at the interface between the membrane and the aqueous compartment – i.e. at the lipid headgroups. With the development of synchrotron insertion devices, boosting the available beam intensities by orders of magnitude, the quality of X-ray reflectivity measurements has also been dramatically improved by almost doubling the accessible  $Q_z$  range. Similarly, the available  $Q_z$  range in surface-sensitive neutron scattering has been continuously extended owing to a careful sample cell design (Krueger *et al.*, 1995, Meuse *et al.*, 1998), and by boosting the available neutron flux, the commissioning of next-generation neutron sources will drive this development even further (Blasie and Timmins, 1999).

These developments resulted in a considerable gain in resolution of the underlying structures – and required a reexamination of the models used in the past for the evaluation of reflectivity data. While the so-called two-box model, that has been extensively used to describe the submolecular organization of (phospho-) lipid surface monolayers (Helm *et al.*, 1987, Möhwald *et al.*, 1990, Daillant *et al.*, 1991, Helm *et al.*, 1991, Vaknin *et al.*, 1991, Rieu *et al.*, 1995), has been very well capable of describing X-ray data sets that extend up to  $Q_z^{\max} \sim 0.5 \text{ \AA}^{-1}$ , such models fail at the description of the high-resolution data available to date (Weygand *et al.*, 1999). In response, novel data modeling strategies have been developed (Wiener and White, 1991b, Schalke *et al.*, 2000b) that use distribution functions to describe the organization of submolecular fragments, particularly of phospholipid headgroup substructures, normal to the interface. Such a development has been greatly facilitated by the recent availability of both model-free data inversion techniques (Zhou and Chen, 1993, Hamley and Skov Pedersen, 1994, Skov Pedersen and Hamley, 1994b, Skov Pedersen and Hamley, 1994a, Berk and Majkrzak, 1995, Chou *et al.*, 1997) – enabling an analysis of the inherent weaknesses of the two-box approaches – and volumetric information on lipid substructures from molecular modeling (Petrache *et al.*, 1997, Armen *et al.*, 1998) – used to reduce the uncertainties in the complex distribution-function model.

Lateral correlations of in-plane structural properties are tested by nonspecular scattering of X-rays. Within the distorted-wave Born approximation (DWBA), the observed intensity,  $I$ , is related to the structure factor of a rough surface,

$$S(Q) = \frac{\exp(-1/2|Q_z|^2 \sigma^2)}{|Q_z|^2} \iint (\exp(|Q_z|^2 C(R)) - 1) \exp(i(Q_x x + Q_y y)) dx dy \quad (4)$$

which is characterized by height-height correlations,  $C(R) = \langle z(0)z(R) \rangle$ , by (Sinha *et al.*, 1988)

$$I \propto |T_i(Q)|^2 |T_f(Q)|^2 S(Q) \quad (5)$$

where  $T_{i/f}$  are the Fresnel transmission functions.

## II. DATA INVERSION AND MODELING IN REFLECTIVITY MEASUREMENTS

Biological membranes are generally fluid systems with a high degree of in-plane disorder. Thus, in-plane diffraction methods are often not suited to characterize the physiologically relevant states of such systems. Reflectivity measurements, on the other hand, are sensitive to the electron density profile across an interface and are thus perfectly suited to determine the structural organization of laterally disordered interface films. The optics of X-rays and neutron beams at surfaces have been extensively dealt with in the literature (Als-Nielsen and Kjaer, 1989, Penfold and Thomas,

1990, Als-Nielsen *et al.*, 1994). Briefly, the real part of the refraction index  $n$  is slightly different from unity – and usually *lower* – for the relevant frequencies, and the imaginary part of  $n$ , *i.e.*, the absorption coefficient  $\beta$ , is negligibly small,

$$n = 1 - \delta + i\beta \quad (\text{with } \beta \approx 0) \quad (6).$$

This implies *external* total reflection of a plane wave impinging on a planar, ideally sharp interface between two media with a refractive index of 1 and  $n$ , respectively, as long as the incident angle  $\alpha$  is below the critical angle

$$\alpha_c = \sqrt{2\delta} = \frac{\sqrt{4\pi\rho_{av}}}{k} = \begin{cases} \frac{\sqrt{2\pi\rho_{el}r_0}}{k} & \text{for X-rays} \\ \frac{\sqrt{4\pi\rho_n}}{k} & \text{for neutrons} \end{cases} \quad (7).$$

Here,  $k = 2\pi/\lambda$  is the magnitude of the wavevector,  $\rho_{av}$  is the average scattering length density (SLD) of a molecular-size volume  $V$  at the interface,  $\rho_{el}$  is electron density,  $r_0 = \frac{e^2}{m_e c^2} \approx 2.82$  fm is the classical Thomson electron radius and  $\rho_n = \frac{1}{V} \sum v_i b_i$  is the neutron SLD, as computed from the atomic content of  $V$  ( $v$  atoms of the species  $i$  that is characterized by the neutron scattering length  $b$  are contained in  $V$ ). While for X-rays,  $\alpha_c$  is always real, it can be imaginary in neutron experiments, *e.g.*, if the beam is reflected from the surface of H<sub>2</sub>O (for which  $\rho_n < 0$ ). In this case, there is no  $\mathcal{Q}_z$  regime of total external reflection. Since  $\delta$  is of the order of  $10^{-5}$ ,  $\alpha_c$  is typically in the millidegree range.

If one is interested in specular reflectivity, the momentum transfer,  $\vec{Q} = \vec{k}_{out} - \vec{k}_{in}$ , is strictly normal to the interface,

$$\mathcal{Q}_z = 2k \sin \alpha = \frac{4\pi}{\lambda} \sin \alpha \quad (8),$$

where in a local coordinate system  $\hat{x}$  and  $\hat{y}$  define the interface and  $\hat{z}$  points to the perpendicular direction. Hence,

$$\mathcal{Q}_c = \sqrt{4\pi\rho_{av}} \quad (9)$$

is a quantity characteristic of the medium, since it is independent of the wavelength  $\lambda$  (for example,  $\mathcal{Q}_c^{\text{X-rays}} \approx 0.0217 \text{ \AA}^{-1}$  for water). From Fresnel's law, neglecting differences between different polarization directions and higher orders in  $\delta$  which is both justified as  $\delta \approx 10^{-5}$ , one finds a reflection amplitude  $r_{1,2}$  between media with the indices  $n_1$  and  $n_2$

$$r_{1,2} \approx \frac{\alpha - \sqrt{\alpha^2 + 2(\delta_1 - \delta_2)}}{\alpha + \sqrt{\alpha^2 + 2(\delta_1 - \delta_2)}} \approx \frac{\mathcal{Q}_{c,1}^2 - \mathcal{Q}_{c,2}^2}{4\mathcal{Q}_z^2} = \frac{4\pi}{\mathcal{Q}_z^2} (\rho_{av,1} - \rho_{av,2}) \quad (10)$$

and a reflectivity

$$R = |r|^2 \quad (11).$$

The Fresnel reflectivity  $R_F$  of an ideal surface in vacuum (or air) derives from Eqns. (10) and (11) as

$$R_F \approx \left( \frac{Q_c}{2Q_z} \right)^4 \quad (11a),$$

as long as  $Q_z$  is sufficiently large (*e.g.*,  $> 5 Q_c$ ).

In reality, interfaces between two media are not mathematically sharp but graded on the Ångstrom length scale, due to atomic roughness in the case of planar solid state surfaces or thermally excited capillary waves in the case of fluid surfaces (Meunier, 1987).<sup>\*</sup> This is phenomenologically taken into account by convolution of the step function  $\Theta(z - z_0)$ , that describes the ideal interface, with a Gaussian yielding the error function as the relevant profile describing the interface (Als-Nielsen and Möhwald, 1991):

$$\Theta(z - z_0) \rightarrow \frac{1}{2} \operatorname{erf} \left( \frac{z - z_0}{\sqrt{2\sigma}} \right) + \frac{1}{2} \quad (12),$$

where  $\operatorname{erf}(z) = \frac{2}{\sqrt{\pi}} \int_0^z e^{-t^2} dt$  and  $\sigma$  is a parameter related to the amplitude of the roughness. In comparison with the reflectivity from an ideal interface, the surface roughness (s.r.) leads to a Debye-Waller-like damping of the reflection amplitude (Nénot and Croce, 1980),

$$r(\text{w/s.r.}) = r(\text{w/o s.r.}) \cdot e^{-\frac{Q_z^2 \sigma^2}{2}} \quad (13).$$

If a molecularly thin homogeneous film (index  $n_2$ ) is located on a semi-infinite substrate (index  $n_3$ ), reflection according to Eq. (10) occurs at both the front and back faces, giving rise to interference with an intensity pattern in the far field characteristic of the index and the thickness  $d_2$  of the film. The interference originates from a phase factor that takes into account the propagation of the wave in the medium

$$r_{1,2} = \frac{r_2 + r_3 \cdot \exp(2ik_{z,n_2} d_2)}{1 + r_2 r_3 \cdot \exp(2ik_{z,n_2} d_2)} \quad (10a),$$

and the absolute square yields the reflectivity,

$$R = \frac{r_2^2 + r_3^2 + 2r_2 r_3 \cdot \cos(2k_{z,n_2} d_2)}{1 + r_2^2 r_3^2 + 2r_2 r_3 \cdot \cos(2k_{z,n_2} d_2)} \quad (10b).$$

Stratified surface films can obviously be accounted for by recursive application of Eqns. (10), taking into account a global surface roughness by means of Eq. (13), as first suggested by Parratt (1954). Arbitrary SLD profiles at the interface may either be treated by slicing the profiles into a sequence of thin layers and determining the reflectivity by the Parratt recursion algorithm or by the kinematic approximation (Als-Nielsen and Kjaer, 1989, Als-Nielsen and Möhwald, 1991, Als-Nielsen *et al.*, 1994),

$$\frac{R(Q_z)}{R_F(Q_z)} \approx \frac{1}{\rho_{\text{substrate}}^2} \left| \int \frac{d\rho(z)}{dz} e^{iQ_z z} dz \right|^2 \quad (14),$$

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<sup>\*</sup>A rigorous theoretical assessment of the liquid-vapor interface of simple fluids has been recently developed in Mecke and Dietrich (1999).

which is only valid for  $Q_z > 5 Q_c$  but has the advantages of allowing for any analytical form of  $\rho(z)$  while being at the same time more intuitive than the recursion formalism.

As in any scattering experiment, the experimental data cannot be directly translated into the underlying structure because of the "phase problem", *i.e.*, the loss of phase information upon obtaining the scattered intensities from the amplitudes by using either of Eqns. (10) or (14). Data quality and resolution, as well as recent developments in the modeling of membrane surfaces have been recently reviewed (Schalke and Lösche, 2000). For modeling of both x-ray and neutron reflectivity data, "box" or slab models (Als-Nielsen and Kjaer, 1989, Möhwald, 1990, Als-Nielsen and Möhwald, 1991, Als-Nielsen *et al.*, 1994) that describe a lipid monolayer as two contiguous slabs, one hydrophobic and one hydrophilic, have frequently been used to describe aqueous surface monolayers (Helm *et al.*, 1987, Grundy *et al.*, 1988, Kjaer *et al.*, 1989, Bayerl *et al.*, 1990, Helm *et al.*, 1991, Vaknin *et al.*, 1991, Brumm *et al.*, 1994) or water/oil interfaces (Thoma *et al.*, 1996) as well as protein or polymer interactions with lipid surface layers (Johnson *et al.*, 1991b, Vaknin *et al.*, 1991, Lösche *et al.*, 1993, Naumann *et al.*, 1996, Fukuto *et al.*, 1997, Gallant *et al.*, 1998, Gidalevitz *et al.*, 1999a,b, Kuhl *et al.*, 1999, Weygand *et al.*, 1999). Analogous models for data inversion have also been used to describe molecular layer systems at solid/fluid interfaces (Johnson *et al.*, 1991a, Schmidt *et al.*, 1992, Fragneto *et al.*, 1995, Krueger *et al.*, 1995, Diederich and Lösche, 1997, Malik *et al.*, 1997, Kuhl *et al.*, 1998).

More recently, it has been reported that the simple box approach is rather inadequate for the description of surface monolayers from data collected at third-generation synchrotron sources (Frahm *et al.*, 1995) at high momentum transfer (Schalke *et al.*, 2000a, Schalke *et al.*, 2000b, Schalke and Lösche, 2000, Krüger *et al.*, 2001), and a more sophisticated model that describes the interface film in terms of volume-restricted distribution functions (VRDF) across the interface has been developed (Schalke *et al.*, 2000b). The novel data refinement technique is inspired by ideas by Wiener and White (Wiener *et al.*, 1991, Wiener and White, 1991a,b, 1992a,b) for the evaluation of X-ray and neutron small angle scattering from planar multi-bilayer systems. In addition, it takes into account recent developments in molecular dynamics simulations of bilayers (Petrache *et al.*, 1997, Armen *et al.*, 1998), from which volumetric information has been derived and utilized to interpret the small angle scattering data with a higher confidence level. In its implementation for floating surface monolayers, the VRDF model treats the aliphatic chains just as they are accounted for in the box-model approach; the phospholipid backbone and lower headgroup, however, is parsed into fragments that are placed into distribution functions along the interface normal (Schalke *et al.*, 2000b). In distinction from the box model, which satisfies space-filling automatically within the chemical approach to its interpretation, the filling of space has to be explicitly taken into account in the VRDF approach, since the distribution functions may partially or entirely interpenetrate each other and even if they are separated along  $z$ , space-filling is usually not fulfilled at their perimeters. In addition to a satisfactory description of the experimental data, this requires

$$\sum_{\xi} n_{\xi}(z) \cdot V_{\xi} = 1 \quad (15),$$

where  $n_{\xi}(z) = N_{\xi}(z) / (A_{lipid} \cdot dz)$  is the number density of the fragment  $\xi$  within the plane located at a distance  $z$  from the interface and  $V_{\xi}$  is its partial volume. Handling of this additional constraint obviously requires *a priori* knowledge of  $V_{\xi}$ , or their determination from the fit.

Thermal broadening of the interface structure in the distribution function model derives from two contributions that are well distinguished: A broadening by capillary waves,  $\sigma_{cw}$ , and an intrinsic broadening of fragment positions,  $\sigma_{int}$ , which is the value one would expect to observe *without* capillary waves, *i.e.*, within an interface film at an ideally flat surface. If one assumes that typical wavelengths for the two processes are well-separated in real space, the upper and lower interfaces

of the alkane slab – considered to be atomically flat, at least for the ordered phases LC and SC – are *only* affected by  $\sigma_{cw}$ , whereas the distributions of the headgroups fragments are affected by both contributions which are summed geometrically:

$$\sigma_{total,\xi} = \sqrt{\sigma_{cw}^2 + \sigma_{int,\xi}^2} \quad (16).$$

This separation of the contributions to interfacial broadening permits one both to determine the evolution of intrinsic broadening along the isotherm and to check whether the capillary wave’s amplitudes depend on  $\pi$  and  $T$  as predicted by theory (Pershan, 1990, Pershan, 2000, Plech *et al.*, 2000):

$$C(R) = \frac{k_B T}{2\pi\gamma} \cdot K_0\left(R\sqrt{\delta\rho g\gamma}\right) \quad (17).$$

Here,  $k_B$ ,  $\gamma$ , and  $\delta\rho$  denote the Boltzmann constant, surface tension, and mass density difference between fluid subphase and its vapor phase, respectively.  $K_0$  is the zero-order modified Bessel function of the second kind.

### III. CHARACTERIZATION OF PLANAR LIPID MODEL SYSTEMS

#### A. Surface monolayer systems

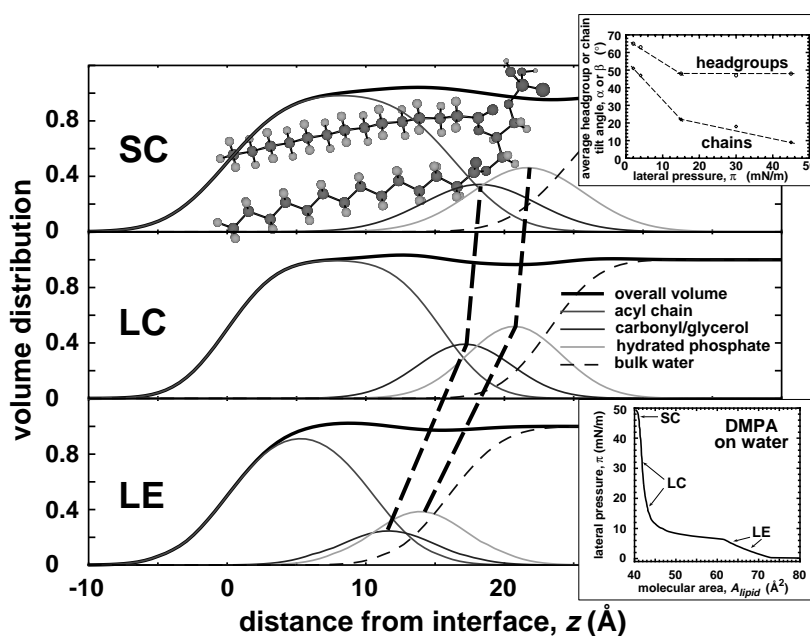
##### 1. Pure lipid monolayers

Monomolecular (phospho-)lipid surface layers (Möhwald, 1990, McConnell, 1991, Knobler and Desai, 1992, Möhwald, 1995) have been intensively studied for their unique properties as quasi 2D molecular systems and the ease by which their molecular properties may be controlled. A rich phase diagram has been established (Kaganer *et al.*, 1999) – primarily by characterizing the order of the amphiphiles’ aliphatic chain using GIXD (Kaganer *et al.*, 1999) and refining this assessment with optical microscopy techniques (Fischer *et al.*, 1995, Teer *et al.*, 1997, Marshall *et al.*, 2000) – that is fairly well understood in terms of Landau theory with a quite limited number of translational and orientational order parameters (Kaganer and Loginov, 1995, Kaganer and Osipov, 1998). It has thus been recently established in great detail, that molecular order within Langmuir monolayers is primarily driven by van-der-Waals interactions between the linear alkane moieties and is controlled by a subtle balance between the packing properties of the chains and the headgroups (Weidemann *et al.*, 1998, Weidemann *et al.*, 1999). Since lipid bilayer membranes may be regarded as a set of two weakly coupled monolayers facing each other, such studies are of potential interest for the comprehension of biologic systems. On the other hand, however, as biological membranes possess a large degree of in-plane disorder, the crystalline and semi-crystalline phases observed in the monolayers are of somewhat limited interest.

While diffraction from the hydrophilic lipid headgroups within monolayers has not been observed as of yet, it is generally assumed that they do not participate in lipid ordering. Nevertheless, since monolayers are regarded relevant model systems particularly for the interface of biomembranes with its aqueous environment, the structural and dynamic properties of the headgroups are of particular interest. Thus, numerous reflectivity studies have been undertaken to elucidate the structure of this interface on a submolecular level. For their interpretation, box models (Als-Nielsen and Kjaer, 1989) provide a relatively simple, rather intuitive approach to data interpretation in submolecular terms with the additional benefit of their easy implementation (Parratt, 1954). This is why they have been extensively used in the past for the inversion of X-ray and neutron reflectivity data in studies of lipid monolayers at aqueous surfaces (Helm *et al.*, 1987, Grundy *et al.*, 1988,

Kjaer *et al.*, 1989, Bayerl *et al.*, 1990, Helm *et al.*, 1991, Vaknin *et al.*, 1991, Brumm *et al.*, 1994, Vaknin and Kelley, 2000) or water/oil interfaces (Thoma *et al.*, 1996), as well as peptide or polymer interactions with lipid surface layers (Johnson *et al.*, 1991b, Vaknin *et al.*, 1991, Lösche *et al.*, 1993, Naumann *et al.*, 1996, Fukuto *et al.*, 1997, Gallant *et al.*, 1998, Gidalevitz *et al.*, 1999a,b, Kuhl *et al.*, 1999, Weygand *et al.*, 1999). Analogous models for data inversion have also been used to describe the structure of (inside-out oriented) opposing monolayer pairs within NBF's (Cuvilliers *et al.*, 2000a).

One potential problem in the data inversion with box models is a breakdown of the chemical interpretation if the local SLD in the real structure shows large variations along the  $\hat{z}$  direction *within one box*. Before the arrival of third-generation synchrotron sources, such variations could not be experimentally determined – due to the lack of resolution – and may thus have resulted in misinterpretations of the atomic contents of the boxes since the interface between them is not naturally determined by chemical composition but rather by the gradients of the SLD, *c.f.* Eq. (14). The steepest gradient within a phospholipid film in which the headgroup extends substantially into the subphase needs not be located at the hydrophilic/hydrophobic interface but may occur closer to the phosphate moiety – in which case the chemical interpretation of the headgroup slab as one homogeneous structure may be dangerously misleading. In this context, one may challenge earlier results on the structural reorganization of DMPE and DLPE monolayers upon monolayer compression – *i.e.*, reduction of the area per lipid molecule in the surface film,  $A_{lipid}$  – (Möhwald, 1990, Helm *et al.*, 1991) for which it has been reported that the hydration number,  $n_w$ , changes from  $\sim 24$  at  $A_{lipid} \sim 80 \text{ \AA}^2$  to  $\sim 1.5$  for DMPE at  $40 \text{ \AA}^2$  while undergoing the main monolayer phase transition,  $LE \rightarrow LC^*$ . Similar dramatic changes in phospholipid headgroup hydration at the  $LE/LC$  phase transition were reported for DPPC as observed by analyzing box models describing the neutron reflectivity of DPPC monolayers (Naumann *et al.*, 1995). The results from these box model studies have in common that the apparent thickness of the headgroup layer,  $d_{hphib}$  is as large as – or



**Figure 2:** Volume-restricted distribution function approach for the modelling of planar lipid layer systems as exemplified for DMPA on water (data from Schalke *et al.*, 2000b). Dashed fat lines in the main panel indicate the development of the center locations of the fragment distributions upon film compression from which the average headgroup tilt angle,  $\alpha$ , is deduced. Insets indicate the surface isotherm (bottom) and the concurrent tilt of the lipid headgroups ( $\alpha$ ) and chains ( $\beta$ ) toward the surface normal upon compression. The  $LE \rightarrow LC$  phase transition shows clearly in the development of the tilt angles.

\*Phospholipid monolayer phases are denoted using the nomenclature introduced by Cadenhead *et al.* (1980): G, gaseous; LE, liquid expanded; LC, liquid condensed; SC, solid condensed

even larger than ( $> 11 \text{ \AA}$ ) – the extended length of the headgroup fragments, particularly in the disordered phases, and that  $d_{hphil}$  decreases upon reduction of  $A_{lipid}$ . That would imply a tilting movement of the headgroup dipoles away from the surface normal while the average chain directions tilt toward the surface normal upon reduction of the available area per molecule. Moreover, the apparent value of  $d_{hphil}$  was invariably close to, or even larger than, the extended length of the PE headgroup. Together with the notion that only 1.5 water molecules should hydrate the phosphate in a compressed monolayer *on top of an infinite reservoir of water*, these results raise severe doubts about the validity of the model. A careful examination of this counterintuitive situation by the authors of the first study concluded with the statement that *within the two-box model approach* results were robust against variations of details of the model (Helm *et al.*, 1991).

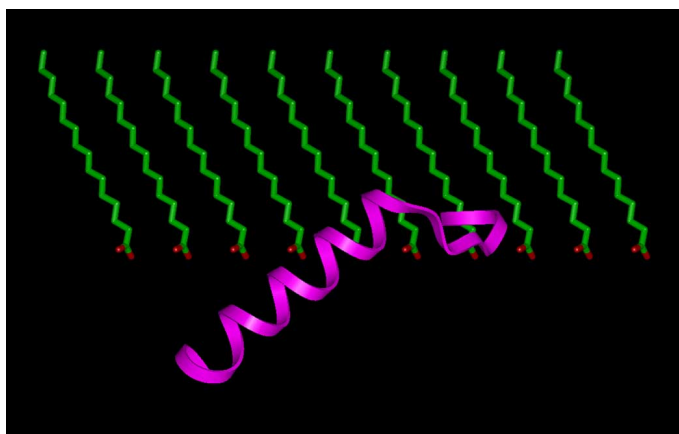
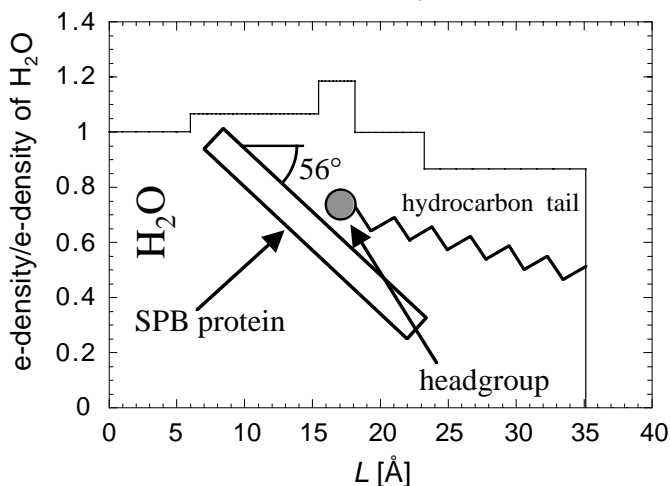
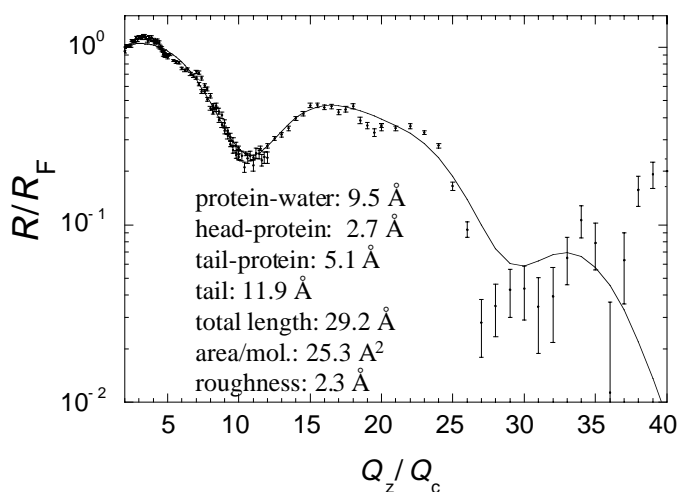
With the development of the VRDF approach to reflectivity data evaluation, more intuitive and more consistent results on the submolecular structure of phospholipid monolayers begin eventually to appear (Schalke, 2000, Schalke *et al.*, 2000b, Schalke and Lösche, 2000, Krüger *et al.*, 2001). For a number of distinct phospholipids it has been reported that the phosphate hydration is constant along the isotherm – number of water molecules associated with the phosphate,  $n_w^p \sim 5 \dots 6$  (Schalke *et al.*, 2000b, Krüger *et al.*, 2001) – while the decrease of the average chain tilt angle from the surface normal,  $\beta$ , is accompanied with a corresponding decrease of the average headgroup tilt, *c.f.* Fig. 2. For the two anionic phospholipid headgroups, phosphatidic acid (PA) and the larger phosphatidylglycerol (PG), it has been reported that an unexpectedly large amount of ions is bound on concentrated salt solutions (10 mM  $\text{BaCl}_2$ ) which are continuously squeezed out of the headgroup region upon compression. Only at high surface pressures,  $\pi$ , the expected stoichiometric ratio of 2:1 ( $\text{PA}^-:\text{Ba}^{2+}$ ) has been observed. In both cases it was observed that  $\alpha$  stays essentially constant over a large range of  $\pi$  while the decrease of  $A_{lipid}$  is compensated for by the exclusion of ions (Schalke and Lösche, 2000, Krüger *et al.*, 2001). Consistent with intuitive expectation, the value of  $\alpha$  is greater for the small PA headgroup ( $\alpha \sim 35^\circ$  in the condensed phase on 10 mM  $\text{BaCl}_2$ , Schalke and Lösche, 2000) than for the PG headgroup ( $\alpha \sim 25^\circ$  under the same nominal conditions, Krüger *et al.*, 2001). In distinction,  $\alpha \sim 65^\circ$  and  $45^\circ$  has been observed for PA on pure water subphase in the LE and LC phases, respectively (Schalke *et al.*, 2000b). In any of these cases, hydration of the carbonyl moieties is small as was thus neglected in the models. The distribution width of the phosphate position as projected on the surface normal was found to be significant in all these structures (FWHM width,  $\sigma_{int} \sim 2 \text{ \AA}$ ) and was generally observed to increase upon compression. It appears thus that a fair amount of headgroup disorder characterizes the structure of such monolayers.

One important caveat that applies to all of the models for phospholipids with headgroups more complex than the simplest (PA) is that the terminal moiety, such as the secondary glycerol in PG or the choline in PC is quite similar in electron density to the surrounding water. This implies that these models are relatively vulnerable since they are based entirely on X-ray reflectivity results at this point, and a more definite structural assessment will have to await comprehensive neutron reflectivity measurements on headgroup-deuterated lipids.

Contrast variation using X-ray synchrotron radiation, on the other hand, is feasible as recently demonstrated at the 6-ID beamline of the APS (Argonne National Laboratory): In an exploratory experiment, the anomalous scattering of  $\text{Ba}^{2+}$  ions due to their L<sub>I-III</sub> absorption lines has been utilized to characterize the binding of such ions to anionic DMPA headgroups in great detail and without any assumptions (Schalke and Lösche, 2000) on the charge distribution at the interface (Krüger, Vaknin and Lösche, unpublished results). Such experiments should pave the way, *e.g.*, for detailed studies of ion binding to lipid membranes or the molecular mechanisms of biomineralization at molecularly well-defined model interfaces (Lochhead *et al.*, 1997).

## 2. Composite monolayer systems

Composite surface monolayer systems have been frequently studied to better comprehend the various interactions of peptides (and other macromolecular components of biological relevance) with membranes (Möhwald, 1990, Möhwald *et al.*, 1995). Of particular significance are such systems that form monolayers in their physiological environment, such as pulmonary surfactant models (Lee *et al.*, 1997, Galla *et al.*, 1998, Krüger *et al.*, 1999), or that mimic the association of peripheral ligands, such as components of the cellular exoskeleton (Sackmann, 1995) or membrane fusion mediators (Arnold, 1995, Gerke and Moss, 1997). Other studies concern the action of phospholipases at membrane surfaces and address particularly the interdependence of lipid order and enzymatic activity. Finally, a number of studies are dedicated to the elucidation of the surface-dependence of peptide folding (White and Wimley, 1998) and of surface-induced peptide or protein crystallization processes (Kühlbrandt, 1992, Brisson *et al.*, 1999).



Pulmonary surfactant is one of the rare cases, where a monomolecular lipid layer is the equivalent of the physiological system. However, during the respiration cycle, this monolayer undergoes a reversible, peptide-mediated transformation into a state of (what is believed to be) higher organizational order. Particularly the surfactant peptides, SP-B and SP-C, are implicated in the refinement of the lipid mixture that involves a high percentage of PC's and PG's and has an unusually large proportion of saturated chains. In fact, this is the only known example where DPPC seems to be a major component of a physiological system. Recently, an explicit model of the topological changes and a mechanism that involves the hydrophobic SP fractions has been proposed (Galla *et al.*, 1998). A particular model system, phosphatidic acid (PA) and a truncated form of synthetic SP-B (SP-B<sub>1-25</sub>) that has been extensively studied (Lipp *et al.*, 1996) as a replacement formulation of natural pulmonary surfactant for the treatment of acute respiratory distress syndrome (ARDS), has recently been characterized using X-ray scattering (Lee *et al.*, 2000, 2001). In connection

Figure 3: Association of the recombinant, truncated surfactant peptide B (SP-B<sub>1-25</sub>) with palmitic acid monolayers according to Lee *et al.* (2001). (a) X-ray reflectometry data and (b) deduced SLD profile from a box modelling approach. (c) Molecular model of the complex surface monolayer.

with the molecular structure of the peptide, as it appears from FTIR spectroscopy and molecular dynamics simulations (Gordon *et al.*, 2000) – a rather hydrophobic  $\alpha$  helix adjacent to a short (7 AA)  $\beta$  structure at the N-terminus that is believed to promote the incorporation of the peptide into the monolayer (Hawgood *et al.*, 1998), it was deduced from the electron density profile using a box model that the peptide, which was included at 20 wt% – well above the physiological concentration of SP-B –, intercalates the headgroup and is oriented under an oblique angle, as visualized in Fig. 3. By and large, however, the results point to differences of the mesoscopic organization of the surfactant film upon interaction of the peptide rather than changes at the molecular level.

Another field of intense research concerns the molecular-level structure and molecular interactions of components of the cytoskeleton with the lipid membrane. Neutron reflection measurements have been conducted to reveal particularly the structural features of spectrin (Johnson *et al.*, 1991b), hisactophilin (Naumann *et al.*, 1996), or actin filament (Demé *et al.*, 2000) binding to floating monolayers. At the current stage, as long as box modeling still prevail as the main tool for data inversion, these studies are essentially limited to determining whether or not the peptides bind in homogeneous layers at the interface, quantifying apparent layer thickness values (and comparing those with dimensions of the respective protein molecules) as well as equivalent volume occupation of the peptide in the surface-adsorbed layer, and optimizing experimental conditions for peptide binding. Particularly the most recent paper (Demé *et al.*, 2000) reports a comprehensive study on the ionic strength dependence of the electrostatic interaction of a peptide (F actin) with charged interfaces.

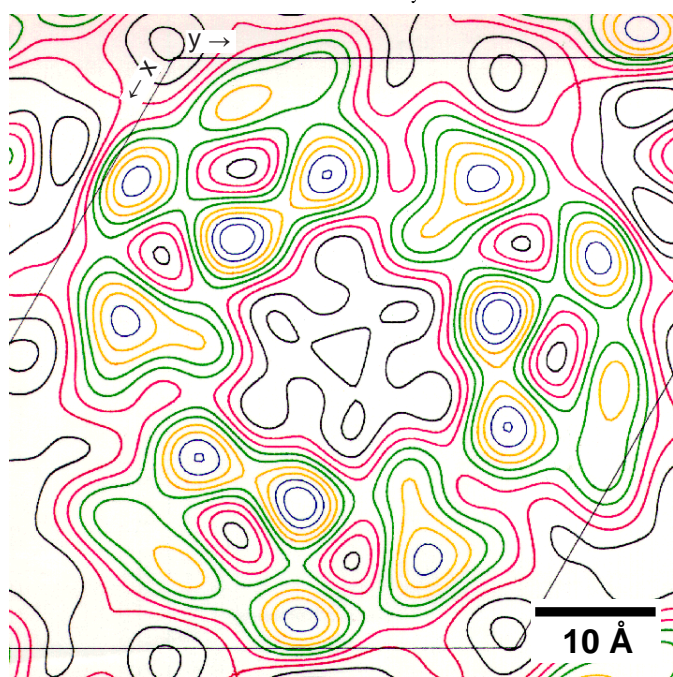
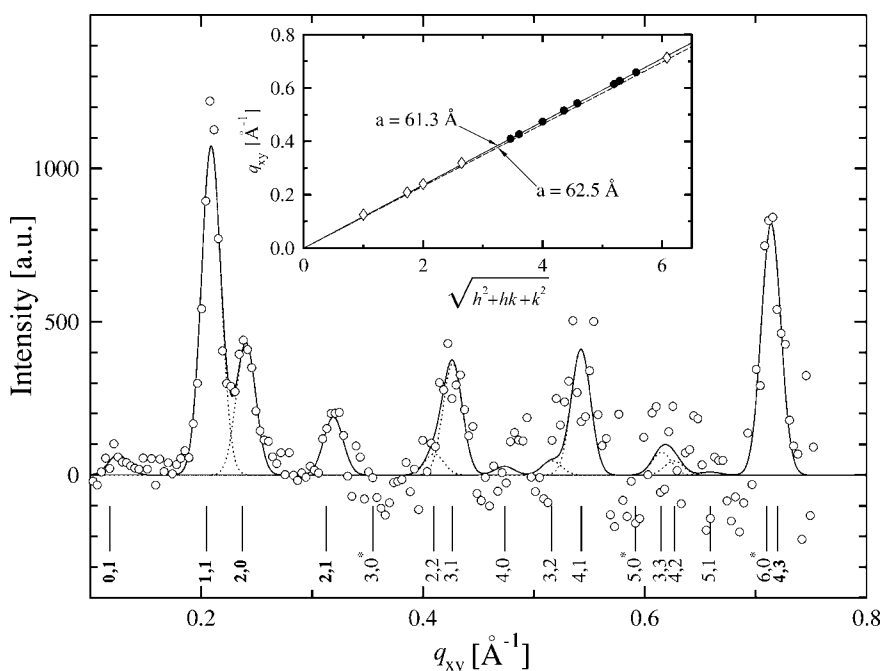
Annexins are a class of proteins known to promote vesicle aggregation and fusion *in vitro* in a  $\text{Ca}^{2+}$  dependent manner and implicated in membrane trafficking (Gerke and Moss, 1997). Although they have been extensively studied in recent year, little is known about the molecular details of their membrane interaction. A recent study addresses such questions by characterizing the membrane binding of annexin I in X-ray reflectivity measurements (Bitto *et al.*, 2000). Since annexin I has two inequivalent membrane binding sites, a number of different scenarios for the mediation of close contact between two fusing membranes by the protein are viable. Out of these, a mechanism is favored on account of the experimental results in which membrane fusion is promoted by the formation of a laterally aggregated protein monolayer on one membrane surface, in which annexin I is uniaxially attached via its primary binding site, and the subsequent binding of the juxtaposed membrane via interactions with the secondary binding site of the proteins (Bitto *et al.*, 2000).

The interaction of phospholipase  $A_2$  (PLA<sub>2</sub>) with PC monolayers has been studied by Brezesinski and coworkers with a host of surface sensitive characterization techniques including GIXD (Dahmen-Levison *et al.*, 1998, Peters *et al.*, 2000).

Protein unfolding or refolding at aqueous surfaces – neat or covered with lipid films – has been studied by various groups (Gidalevitz *et al.*, 1999a, Gidalevitz *et al.*, 1999b, Holt *et al.*, 2000, Strzalka *et al.*, 2000). Gidalevitz and coworkers studied the denaturation of glucose oxidase (GOx), alcohol dehydrogenase, and urease at bare buffer surfaces and report that the deposition of a Langmuir monolayer does not prevent such denaturation (Gidalevitz *et al.*, 1999a) while crosslinking of GOx with glutaraldehyde does, as assessed by the observation of a protein layer that resembles closely the molecular dimensions of the respective species while denaturation is conjectured from the observation of peptide monolayers whose thickness resembles the approximate diameter of an  $\alpha$  helix and is thus far below the proteins dimensions. Qualitatively similar behavior has been reported for myoglobin at aqueous surfaces (Holt *et al.*, 2000). Strzalka and coworkers studied the association of synthetic  $\alpha$ -helical peptides, so-called maquettes (Robertson *et al.*, 1994), at clean and DLPE-covered buffer surfaces. While the peptides are found to adsorb with their helices coplanar and form coherent peptide layers at low surface pressures they are desorbed into the subphase upon subsequent compression. A variant of the parent maquette that bears hydrophobic alkyl anchors, however, is stabilized at the interface by incorporation of the anchors into a DLPE surface film and

is reported to undergo a transition upon compression in which the coplanar orientation of the helices switches to preferentially normal. No in-plane correlation of the proteins, however, has been reported in any of these cases.

The crystallization of proteins underneath Langmuir monolayers (Darst *et al.*, 1991, Vénien-Bryan *et al.*, 1998) and subsequent characterization at interfaces using GIXD on the other hand is emerging as an effective tool for structural studies in the work of a growing number of groups. The Möhwald group has first reported x-ray diffraction from a monomolecular crystal sheet of streptavidin formed underneath a surface monolayer of a biotinylated lipid (Haas *et al.*, 1995). The monodomain size of such crystal sheets is of the same order as the domain sizes of ordered lipid domains (Krüger and Lösche, 2000),  $\varnothing \sim 10 \mu\text{m}$  (Lösche *et al.*, 1993), and the beam footprint in a typical synchrotron x-ray experiment is of the order of  $1 \text{ cm}^2$ , implying that 2D powder diffraction is observed in these experiments. Working with the well-characterized bacteriorhodopsin (bR) system (Grigorieff *et al.*, 1996, Pebay-Peyroula *et al.*, 1997), Verclas and coworkers have considerably pushed the technology (Verclas *et al.*, 1999). For purple membrane (PM) deposited on a clean

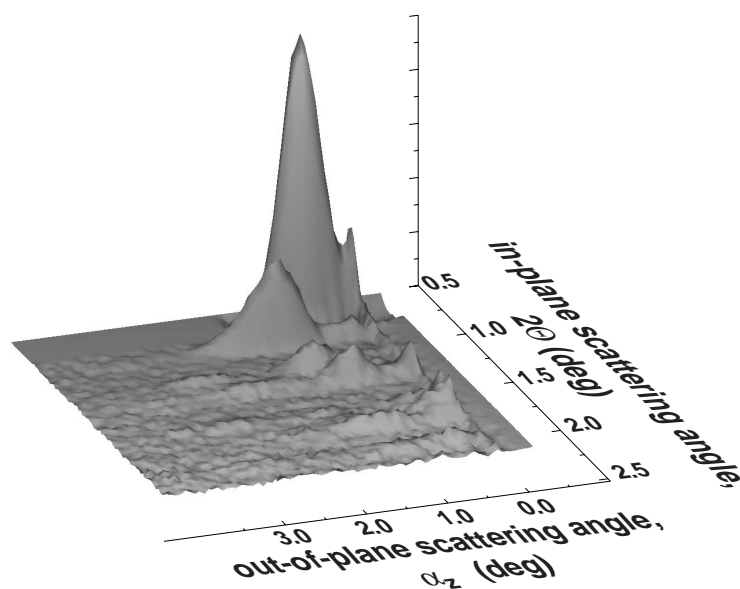


buffer surface, they reported diffraction out to the prominent  $h,k = 4,3$  peak at  $Q_{xy} \sim 0.72 \text{ \AA}^{-1}$  (overlaid with the  $h,k = 6,0$  diffraction peak of the crystal powder) of the hexagonal lattice of the protein/lipid co-crystal, c.f. Fig. 4a. While the same lattice symmetry was observed as in earlier electron microscopy work (Henderson *et al.*, 1990), the lattice constant was reported to be a few percent smaller ( $61.3 \text{ \AA}$  vs.  $62.5 \text{ \AA}$ ) in PM patches deposited at the air/water interface. Generally, this work demon-

**Figure 4:** (a)  $Q_z$ -averaged, background-corrected small-angle GIXD intensity measured for scattering from a PM film at  $\pi = 10 \text{ mN/m}$  on an aqueous surface (250 mM KCl). The Bragg peaks have been indexed under the assumption of a hexagonal lattice with a lattice constant  $a = 62.5 \text{ \AA}$ . The linearity of the  $Q_{xy}$  values of the peak positions vs.  $\sqrt{h^2 + hk + k^2}$ , shown in the inset, verifies the assumption of a hexagonal lattice and quantifies the deviation of the lattice constant from that measured in electron microscopy. Reproduced from Verclas *et al.* (1999) with permission.

(b) Projected electron density map using the intensities shown in (a) and phases derived from electron microscopy (Henderson *et al.*, 1986). The annular structure of the trimeric seven helix bundles within the unit cell is clearly identified.

strates that a resolution is attainable in such studies that approaches 8 Å, such that structural elements such as  $\alpha$  helices can be located within molecular films of crystalline protein, *c.f.* Fig. 4b. At the ESRF, Berge *et al.* have studied GIXD from molecular protein sheet crystals of streptavidin – for which they were also able to attain a maximum in-plane momentum transfer corresponding to a resolution of  $\sim 10$  Å –, annexin V, and the RNA transcription factor, HupR, ligated via an attached multi-histidin tag nickel-chelated to a functionalized lipid headgroup in the surface monolayer (Lenne *et al.*, 2000). For the streptavidin diffraction pattern, Bragg rods have been extracted from the spectra. Similarly, Weygand and coworkers have studied the binding of bacterial S-layer (Sleytr *et al.*, 1999) protomers to phospholipid surface monolayers and their subsequent recrystallization into coherent S-layer lattices (Diederich *et al.*, 1996, Wetzer *et al.*, 1998, Weygand *et al.*, 1999, Weygand *et al.*, 2000) at the HASYLAB BW1 beamline (Frahm *et al.*, 1995) of DESY. For S-layers of the Gram-positive eubacterium, *B. sphaericus* CCM2177, which is the best studied example to date, the coupling of the protein to the lipid monolayer\* and the recrystallized S-layer structure has been studied in detail. Background-corrected Bragg rods determined from the small-angle GIXD of such S-layers recrystallized under a DPPE monolayer are shown in Fig. 5a (Weygand, 2000). In



FTIR and X-ray scattering studies it has been observed that the peptide side chains on top of the attached S-layer interact with the phospholipid headgroups while leaving the acyl chains essentially unaffected (a minor increase in chain order has been reported: Diederich *et al.*, 1996, Weygand *et al.*, 1999). The protein – whose atomic 3D structure is unknown due its apparent incapability to crystallize in three dimensions – forms a porous layer at the lipid that controls transport to the membrane surface. The protein’s volume density distribution normal to the interface has been determined (Weygand *et al.*,

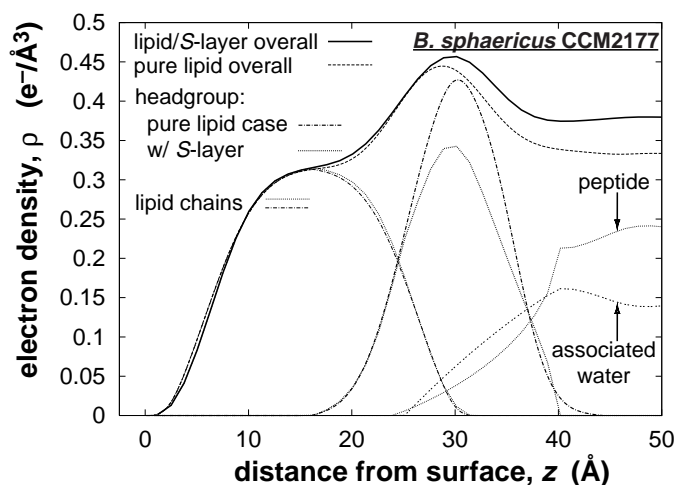


Figure 5: Structural characterization of reconstituted S-layers (*B. sphaericus* CCM 2177) underneath DPPE monolayers on an aqueous subphase containing 10 mM  $\text{CaCl}_2$ . (a) Small-angle GIXD showing the background-corrected Bragg rods deriving from scattering from the monomolecular protein crystal sheet. (b) Peptide insertion into the lipid layer as revealed from a joint model refinement using X-ray and neutron reflectivity data. Both adapted from Weygand (2000).

\*Note that the S-protein/phospholipid interaction is a *non-natural* coupling, as the S-layer resides at the outer face of a *peptidoglycan* cell wall in Gram-positive bacteria (Sleytr *et al.*, 1993). The motivation for studying S-layers in such non-natural supramolecular complexes is their potential for the stabilization of lipid bilayer membranes (Schuster *et al.*, 1998a, Mader *et al.*, 1999).

1999) by using the model-independent data inversion technique of Skov Pedersen and Hamley (1994b). It shows high protein density both close to the lipid membrane surface and at the far face of the porous layer and water-filled cavities near the center of the recrystallized S-layer. From complementing neutron reflectometry measurements on hetero- (lipid/S-layer protein) bilayer systems at various contrasts, the interaction of the peptide with the lipid headgroups emerges in great detail, as shown in Fig. 5b (Weygand, 2000, Weygand *et al.*, 2001). This is consistent with the observation that particularly the asymmetric and symmetric P–O stretch vibrations of the lipid headgroup phosphate, as observed in surface-sensitive FTIR measurements (FT-IRRAS, Mendelsohn *et al.*, 1995), are sensitive to the S-layer recrystallization at the interface (Weygand *et al.*, 2000).

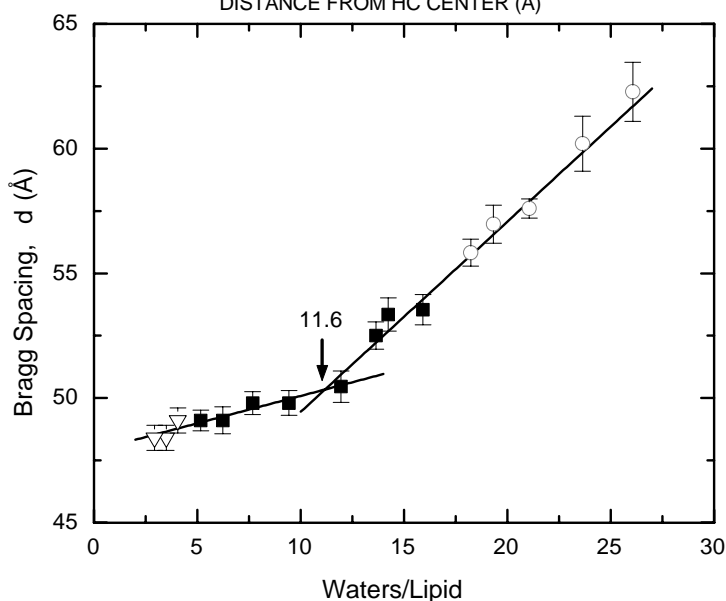
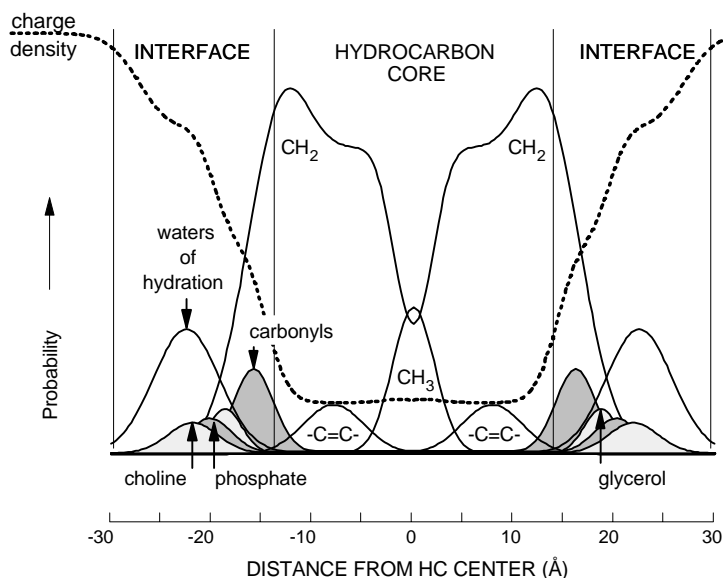
Other recent investigations of protein/lipid systems at the air/water interface include an FTIR and X-ray reflectivity characterization of photosystem II core complex (Gallant *et al.*, 1998), the binding of cytochrome C to mixed, phase separated phospholipid monolayers using neutron reflectivity and FTIR measurements (Maierhofer *et al.*, 2000), and the membrane association of the HIV-1 related accessory protein, virus protein U (Vpu, Marassi *et al.*, 1999), that helps facilitate virus release from infected cells (Zheng *et al.*, 2001). For the latter system it has thus recently been established that the two amphiphatic helices of Vpu located at the C-terminal extend into the sub-phase while its N-terminal hydrophobic helix intercalates the hydrophobic chain slab. The average tilt angle of this helix was observed to depend on surface pressure, and a continuous decrease of the tilt from the surface normal has been reported with increasing pressure. Concomitantly the cytoplasmic helices were found to flatten progressively toward the membrane surface, attributed to a comparably restrictive hinge sequence between the hydrophilic and the first amphiphatic helix. Only at the highest surface pressure studied,  $\pi = 55$  mN/m, the electron density profile indicated the displacement of the C-terminal helix from the monolayer, presumably owing to steric hindrance among the helices at high peptide concentrations at the membrane surface.

## **B. Lipid bilayers and multi-bilayer stacks**

### **1. Pure lipid systems**

Phospholipid bilayers prepared at planar (or macroscopically curved) solid supports and particularly multi-bilayer stacks that may add up to a few  $\mu\text{m}$ 's in total thickness lend themselves for a much more facile, and thus more complete, characterization with the methods covered in this chapter. Exploiting X-ray and neutron diffraction from multi-bilayer stacks on an absolute scale (Franks *et al.*, 1978), Wiener and White have thus perfected the physical characterization of fluid bilayers in a fully resolved, and complete, structural model by determining the transmembrane distribution of individual submolecular fragments (Wiener and White, 1992b and references therein). Such fluid bilayers resemble closely the physical state of actual biomembranes, and particularly the transmembrane distribution of double bonds of the unsaturated chains contained in physiological lipids is a sensitive indicator for the partitioning of peptides into the membrane (Jacobs and White, 1989). These distributions, which represent the time-averaged projection of the molecular fragments on the bilayer normal, may be determined by neutron diffraction from samples containing specifically deuterated fragments (Wiener *et al.*, 1991) or by x-ray diffraction from samples containing specifically brominated lipids that have been shown to be isomorphous for the case of DOPC (Wiener and White, 1991c). From this work, a general picture has appeared that divides the bilayer structure into a hydrocarbon core region,  $\sim 30$  Å thick, and an interface region sandwiching the core on both sides with  $\sim 15$  Å thick slabs that include both the hydrophilic headgroups and membrane-bound water. These two distinct regions may in some sense be treated as two distinct "phases".

A limitation of Wiener/White approach to fluid bilayer structure elucidation is that only samples at reduced hydration, controlled via the relative humidity (RH) of the sample environment, diffract sufficiently well for the determination of a fully resolved picture\* and that the close apposition of neighboring headgroup layers within the multilayer stacks – typically separated by  $\sim 10 \text{ \AA}$  of headgroup-bound water – limits the accessibility of the membrane surface to external solutes. A fully resolved model of such bilayer membranes (Fig. 6a) have hence thus far only been resolved at RH values that correspond to  $\sim 5$  water molecules per lipid (Wiener *et al.*, 1991, Wiener and White, 1992a, Wiener and White, 1992b). This picture has been considerably refined with the help of molecular dynamics simulations (Petrache *et al.*, 1997, Armen *et al.*, 1998). Under-resolved bilayer models at higher hydration have recently been determined up to 93% RH, corresponding to 9.4 water molecules per lipid, using X-ray diffraction from oriented samples and extended into the regime of even higher hydration by X-ray diffraction from unoriented membranes in the form of lipid suspensions (McIntosh *et al.*, 1987, McIntosh *et al.*, 1989) in poly(vinylpyrrolidone) (PVP) solutions (Hristova and White, 1998). The dependence of the Bragg spacing on the sample water content (Fig. 6b) suggests that 12 water molecules complete the hydration shell of the PC headgroups. In connection with the finding from monolayer studies that  $\sim 6$  water molecules hydrate the phosphate within phospholipid headgroups independent of the molecular area within the film (Schalke and Lösche, 2000), this suggests that an extra  $\sim 6$  water molecules are tightly bound at the head-



group adjacent to an (infinite) fluid compartment.

The cross-over from partially to fully hydrated membrane stacks has been recently studied in detail using non-specular neutron and X-ray scattering, which enable the quantification of lateral height-height correlation functions, as well as specular reflectivity (Münster *et al.*, 1999, Salditt *et al.*, 1999,

**Figure 6:** (a) Distribution of sub-molecular lipid fragments across a DOPC bilayer as determined from the joint refinement of X-ray and neutron diffraction. Overlaid as a dashed line is the derived polarity ("charge density") profile for the bilayer structure. Membrane-adsorbed helical peptides, as characterized in X-ray diffraction from bilayer-peptide systems (Hristova *et al.*, 1999), are located in the headgroup region ("INTERFACE") where the steepest gradient of the polarity profile occurs. Based on Fig. 6 of White and Wimley (1999).

(b) Cross-over from partial dehydration to full hydration of phospholipid (DOPC) membrane surfaces as observed in X-ray membrane diffraction. Reproduced from Hristova and White (1998) with permission.

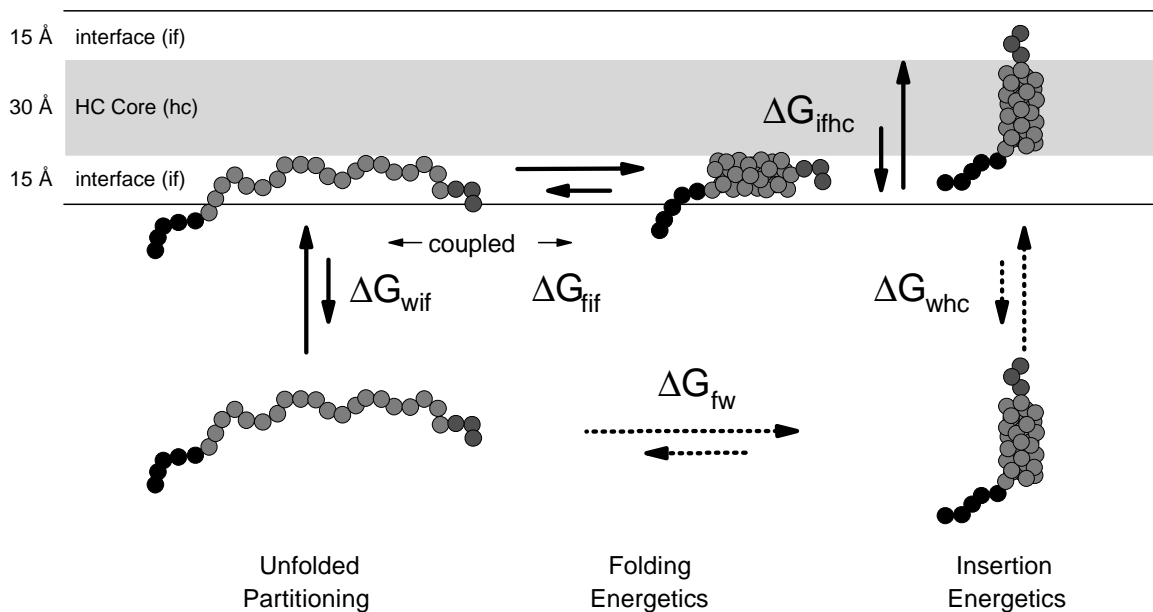
\*At higher hydration, thermal fluctuations degrade the intensities of higher diffraction orders (Zhang *et al.*, 1994).

Salditt *et al.*, preprint). For DMPC multi-bilayer membranes at a RH slightly below 100% it has thus been observed that thermal fluctuations are greatly suppressed, resulting in an absence of the Landau-Peierls effect that is typical of liquid-crystalline order (Als-Nielsen *et al.*, 1980). Thus, significant deviations from the standard Caillé theory (Caillé, 1972) have been observed (Salditt *et al.*, 1999). Rather, it is concluded, that local static defects dominate the line shape of the diffuse intensity both in neutron (Münster *et al.*, 1999) and in X-ray scattering (Salditt *et al.*, 1999). In the course of that work, the unbinding of the multilamellar stacks as a function of temperature has been observed with such reflectivity measurements (Vogel *et al.*, 2000). On the other hand, inelastic X-ray scattering has been utilized to study the collective thermal motion of bilayer stacks (Chen *et al.*, 2001), and a dispersion relation for the acoustic modes has been thus experimentally derived. In this dispersion relation, a softening of the excitation around  $k = 1.4 \text{ \AA}^{-1}$  has been observed that has been implied to play a role in the transport of small molecules, such as  $\text{H}_2\text{O}$ , across the membrane in the liquid crystalline ( $L_\alpha$ ) phase. Quasielastic neutron scattering spectra of two-component (DPPC/cholesterol) membrane stacks have been interpreted as to be indicative of an anisotropic out-of-plane motion of the sterol molecules (Gliss *et al.*, 1999), conceivably similar to a ‘wobbling’ motion of cholesterol implied from biochemical investigations of sterol transfer between membranes (Steck *et al.*, 1988).

Due to the greatly reduced volume density of scattering centers solid supported lipid bilayers are even more difficult to characterize than multi-bilayer stacks. The internal (solid support/lipid membrane) is inaccessible to X-rays in the mid-energy range ( $E_{X\text{-ray}} \sim 10 \text{ keV}$ , corresponding roughly to a wavelength of  $\lambda_{X\text{-ray}} \sim 1.2 \text{ \AA}$ ) that is most easily available and is generally used for the measurements reported thus far. Neutron scattering provides easy access to buried interfaces, however, due to the relatively low brilliance of even the most advanced neutron sources, the information content of reflectivity spectra is severely limited (Schalke and Lösche, 2000). On the other hand, the great flexibility of contrast enhancement via specific deuteration make neutron scattering measurements well worth the effort. Recent years have seen a continuous increase in the performance of neutron scattering experiments (Krueger *et al.*, 1995, Koenig *et al.*, 1996) – rather through a careful engineering of the sample environment primarily in order to reduce incoherent sample background than through an increase of source performance – as well as data evaluation procedures (Vaknin *et al.*, 1991, Wiener and White, 1991b, Koenig *et al.*, 1996, Lösche *et al.*, 1998, Schalke *et al.*, 2000b) that are generally applicable in neutron or X-ray scattering. Thus, the relatively coarse picture that has been developed for PC bilayers formed at a Si wafer surface (Johnson *et al.*, 1991a, Naumann *et al.*, 1995) has been considerably refined in recent work (Koenig *et al.*, 1996), taking account of lateral inhomogeneities of the membrane as assessed by independent AFM characterization. Weakly bound, freely floating bilayers in close vicinity to a bilayer-covered silicon surfaces have been reported (Charitat *et al.*, 1999) and studied with respect to their thermal responses (Fragneto *et al.*, 2001). Such systems are thought to provide an alternate opportunity for the investigation of peptide interactions with bilayer membranes (Fragneto *et al.*, 2000) (see below).

## 2. Peptide/lipid bilayer systems

The structural and thermodynamic principles of membrane protein folding and insertion as well as the basis of their stability within membranes comprise an area that still contain some of the most pressing open questions in structural biology. The problems involved have been recently spelled out in detail in insightful papers by White and Wimley (1998, 1999). In brief, while the thermodynamic stability of soluble proteins is rather straightforward to determine from bulk-phase partitioning measurements, the situation is much more complex for membrane proteins (*c.f.* Fig. 7), since lipid membranes are characterized by thermodynamic properties of their own: They exist in a free energy minimum that derives from small differences between large enthalpic and entropic



**Figure 7:** Thermodynamics of peptide/protein interactions with the interfacial region of bilayer membranes and its significance for the folding of membrane proteins. Reproduced from White and Wimley (1998) with permission.

terms. As a consequence, free energies of partitioning of membrane solutes, such as peptides or proteins, include changes in bilayer free energy deriving from perturbations inferred by the solute. These contributions have collectively been referred to as the ‘bilayer effect’ (Wimley and White, 1993) and have to be discriminated from the hydrophobic effect that arises from the water interactions with the bilayer constituents. The thermodynamic pathway connecting an entirely unfolded polypeptide chain in aqueous solvent and its correctly folded intramembrane protein form involves thus a transient state in which the unfolded peptide is transferred into a prefolded state, mediated by the presence of the membrane interface, rather than consisting of a simple two-step transformation (folding in solution and membrane insertion, *c.f.* Fig. 7, White and Wimley, 1998). Hence, the whole process may be divided into three major intermediate steps, which depend implicitly on the presence of the membrane interface: (i) binding of the unfolded peptide to the interface, associated with the energy term,  $\Delta G_{wif}$ ; (ii) folding of the peptide that resides at the interface into a compact structure ( $\Delta G_{fif}$ ); and (iii) membrane insertion of the folded, interface-bound protein structure ( $\Delta G_{ifhc}$ ). Along this line, studies of the hydrophobic binding of small peptides at bilayer interfaces have revealed an experimental hydrophobicity scale (Wimley and White, 1996), serving as a reference from which  $\Delta G_{wif}$  may be estimated. Structural issues enter the game as far as the association of larger peptides with membrane interfaces and their aggregation into complexes is concerned (Chung and Thompson, 1996). The bilayer interface regime, as discriminated from the hydrophobic core in the assessment of the bilayer structure (Wiener and White, 1992b), has been shown to account for roughly 50% of the total thickness of the thermal bilayer structure (*c.f.* Fig. 6a) and is very well capable of accommodating a folded peptide helix. Of particular importance for the partitioning of peptide into this region is the fact that it consists of a complex mixture of glyceryl, carbonyl, phosphate, choline and water; even some of the methylenes penetrate this region as revealed from the time-averaged distribution of molecular fragments shown in Fig. 6a. The region is thus rich in optional non-covalent interaction sites for peptides attracted to the membrane surface. Thus even peptides attracted to the bilayer membrane primarily by the hydrophobic effect are found located within its interfacial region, not the hydrophobic core, as determined from neutron diffraction (Jacobs and White, 1989). This may be attributed, at least for small peptides, to their inaptitude to form stable *intramolecular* hydrogen bonds.

Another important property of the bilayer's interfacial region that bears important implications for the trapping of structured (*i.e.*, larger) peptide molecules is the fact that this region experiences the steepest part of the charge density gradient, and thus the steepest gradient in polarity, that occurs across the bilayer, *c.f.* Fig. 6a. Consequently, X-ray diffraction measurements on DOPC multilayers at 66% RH containing the ideally amphiphatic synthetic peptide poly-A (5 mol% with respect to total lipid) showed that the  $\alpha$  helix is well aligned with the membrane surface (inclination angle from the surface,  $\gamma < 15^\circ$ ) where it is perfectly trapped within this polarity gradient: the helix center was found located between the center positions of the carbonyl and phosphate moieties and largely overlapping with the glycerol backbone of the PC (Hristova *et al.*, 1999). A very similar result, even on a quantitative scale, was found for mellitin (1 mol%) association with DOPC bilayers (Hristova *et al.*, 2001) while an artificial mellitin dimer derived from the Q25C mutant of the native peptide, although similarly aligned with the membrane, caused such strong perturbations of the bilayer that no satisfactory model for the overall structure was identified. For the native mellitin, on the other hand, model refinement showed that the mellitin helix is in a conformation consistent with the NMR structure (Okada *et al.*, 1994) rather than the X-ray crystal structure (Terwilliger and Eisenberg, 1982a,b).

While Steve White's laboratory has systematically developed the issue of peptide/lipid bilayer interactions in general terms – as summarized above – Huey Huang's lab at Rice University has specifically investigated the mode of action of endogeneous gene-encoded antimicrobial peptides which are key components of the mammalian immune system (Huang, 2000). X-ray and neutron scattering has been extensively used to clarify the molecular organization of model peptides, such as mellitin, alamethicin, gramicidin, magainin, or protegrin at or within planar bilayer membranes. The most interesting question in that area is the origin of the specificity with which lysis of target bacterial cells is achieved – given the relative unspecific nature of peptide/lipid interactions. It has thus been conjectured that such peptide may occur in two states at the membrane: One dormant state in which the molecules are trapped at the membrane surface, conceivably in an arrangement similar to that described above for poly-A (Hristova *et al.*, 1999) and mellitin (Hristova *et al.*, 2001), and an active, lethal state in which the peptides – whose lengths match that of the bilayer width in most cases – are inserted across the membrane and are thought to form pores that mediate cell lysis (Boman, 1994). Thus, using a geometry that is essentially orthogonal to that in GIXD, He *et al.* have studied the in-plane X-ray scattering of gramicidin inserted into DLPC bilayers, organized in multi-bilayer stacks (He *et al.*, 1993a, He *et al.*, 1993b). Harassing the power of neutron scattering in the same geometry, they were able to directly demonstrate pore formation of alamethicin (He *et al.*, 1995, He *et al.*, 1996) in membranes by utilizing the high contrast between the water (D<sub>2</sub>O) filled channels and their membrane surrounding. It was thus determined that eight monomers of the peptide are most likely involved in channel formation (He *et al.*, 1996). Using these tools, experimental evidence for the 'mattress effect' (Mouritsen and Bloom, 1984) of peptide influence on the lipid bilayer has been reported and in-plane correlation between the channels has been detected that originates presumably from the strain field (Aranda-Espinoza *et al.*, 1996) which the gramicidin pores create within the bilayer (Harroun *et al.*, 1999). Interbilayer correlation, finally, has been detected using neutron scattering (Yang *et al.*, 1999) upon moderate dehydration of the multi-bilayer samples, indicating the formation of 3D ordered peptide/lipid samples (Yang *et al.*, 2000), and interpreted to originate due to hydration forces (Yang *et al.*, 1999). A progressively more detailed comprehension of the action of these antimicrobial peptides on membranes, as well as a molecular-scale structure of peptide/membrane assemblies is thus emerging on the horizon.

GIXD has also been applied to study the order of aligned DMPC multi-bilayer membranes and the effect magainin 2 exerts on such membrane models (Münster *et al.*, 2000). However, in this case only the decay of correlations between the acyl chains upon peptide association with the membrane has been reported. More interesting are reports on the diffuse and specular reflectivity

(Koltover *et al.*, 1998) as well as GIXD (Koltover *et al.*, 1999) from highly aligned bR multilayer stacks in which the thermal disintegration of the PM lattice has been studied and a phase diagram indicating the stability of the protein/lipid co-crystal has been derived showing that the thermal denaturation of the protein depends critically on inter-bilayer distance (Koltover *et al.*, 1999). The thermal denaturation of bR within PM multi-bilayer crystals has been successively studied in detail (Müller *et al.*, 2000), and it has been revealed that although long-range correlation is lost, the protein disintegrates only partially and that only some sections of a partially disintegrated secondary structure is exposed to the aqueous phase, which explains the increased sensitivity of the denatured protein to proteolysis upon denaturation. On the other hand, even in the denatured state at temperatures as high as  $T \sim 100^\circ\text{C}$  play peptide/lipid interactions a major role in preventing the system from the total loss of supramolecular organization (Müller *et al.*, 2000).

Other recent studies of interest include neutron scattering studies of feline leukaemia virus fusion peptide (Davies *et al.*, 1998) and of neurokinin A (Darkes *et al.*, 2000) to aligned planar DOPG and DOPC multilayers. Fragneto and coworkers have characterized the structure of single bilayers of DPPC or DPPC/DPPS (9:1) on flat Si wafers and have investigated the impact of a 16 AA peptide, helix III of the antennapedia homeodomain, that has been used as a transmembrane vector (Fragneto *et al.*, 2000). A water layer of 5 Å thickness was observed between the substrate and the bilayer, independent of headgroup composition. After correction for incomplete coverage (Charitat *et al.*, 1999), the revealed bilayer structure was found in line with that observed in earlier work on such systems (Koenig *et al.*, 1996) with an average tilt of the acyl chains around  $40^\circ$  from the surface normal. Upon introduction of the peptide, changes are most pronounced in the headgroup region, and a significant increase of the apparent roughness of the bilayer structure is reported. Due to the macroscopic heterogeneity of the bilayer, conclusions on the peptide's impact remain somewhat vague, however.

A rather quaint system in the context of lipid/peptide interactions, in which the hydrophilic/hydrophobic bilayer sandwich structure manifests itself in an inside-out fashion as a free-standing film in air, has been investigated by Benattar and coworkers in their structural studies of NBF's using X-ray reflectivity (Bélorgey and Benattar, 1991). While the structural assessment of the pure lipid systems (Cuvilliers *et al.*, 2000a) remains on the level of box models, resulting in a similarly unrealistic picture of the headgroup as do box models of Langmuir monolayers (Schalke and Lösche, 2000), protein (BSA) insertion in such systems has been reported (Benattar *et al.*, 1999) and has been observed to lead to the formation of coherent and macroscopically homogeneous peptide layer within the inside-out structure (Cuvilliers *et al.*, 2000b). Whether this system is capable of promoting the 2D crystallization of proteins or leads to new insights in protein/lipid interactions remains to be seen.

### **C. SAM's, HBM's, and grafted tethered lipid bilayers**

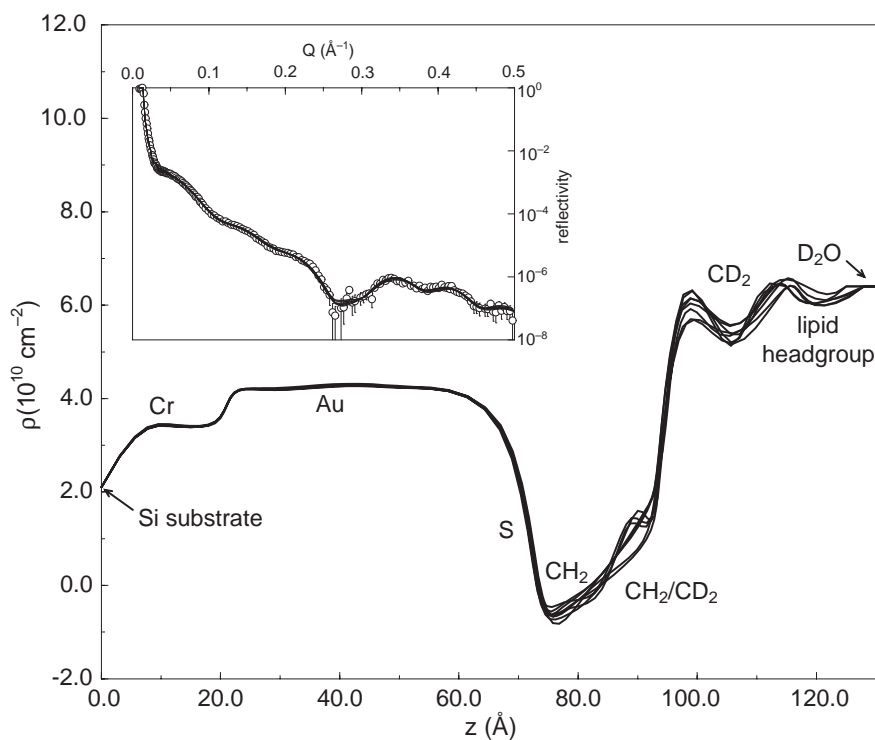
Soft matter functionalized surfaces (Diederich and Lösche, 1997) are generally considered better systems than bare solid state, *e.g.*, Si, Au or glass, surfaces for the preparation of substrate-supported functional, biomimetic peptide/lipid model membranes (Knoll *et al.*, 2000, Sackmann and Tanaka, 2000). Various approaches lend themselves to that end. A general tradeoff exists between the complexity of surface functionalization, resulting in a progressively increasing flexibility for the incorporation of functions into the interface structures, and the level of effort in terms of surface chemistry on the one side and in terms of surface characterization on the other by which the resultant structures may be characterized on the molecular level. Naturally, an aim is thus to achieve advanced and stable functional surface architectures while using minimum effort for the modification.

## 1. SAM's utilized for the implementation of vectorially oriented protein arrays

Organic monolayers, self-assembled and tethered via thiol chemistry to Au or Si surfaces, provide a minimalistic approach for the ‘softening’ of ‘hard’, *i.e.*, solid state, surfaces for the stable functionalization with proteins (Amador *et al.*, 1993). Although not in the center of interest of this review – since no peptide/lipid interactions are involved – the opportunity to prepare organized protein layer structures and the opportunities for their molecular-scale structural characterization is well worth mentioning. Thus, the unspecific binding of human serum albumin (HSA) to hydrophobically terminated SAM's has been studied using X-ray reflectometry, and significant differences in the tenacity of adsorbed layers as a function of the SAM's packing density has been reported (Petrash *et al.*, 1997). If one single AA residue on a protein's surface is utilized for the ligation to the surface, uniaxial orientation may be achieved (Stayton *et al.*, 1992). Reflectivity and interferometry, in which the phasing is achieved through interference of the beam reflected from the surface structure under investigation with a beam reflected at a high-contrast feature buried at a specific position underneath the surface or (neutrons only) at a reference structure prepared with different spin orientations (de Haan *et al.*, 1995, Majkrzak and Berk, 1995, Majkrzak *et al.*, 1998), have been exploited to characterize the association of cytochrome oxidase with SAM surfaces (Edwards *et al.*, 1997, Edwards *et al.*, 1998, Kneller *et al.*, 2001) and the subsequent formation of a bimolecular complex of cytochrome oxidase with cytochrome c (Edwards *et al.*, 1998). Yeast cytochrome c by itself has been immobilized on sulfhydryl-terminated SAM's by its reaction via a naturally occurring, unique surface cysteine residue and its heme site investigated with polarized X-ray absorption fine structure (XAFS) in reflection mode (Edwards *et al.*, 2000). Neutron reflectometry revealed the hydration in terms of water distribution profiles within the protein surface layers (Kneller *et al.*, 2001). SAM's have also been used for interferometry studies of Ca<sup>2+</sup> ATPase (Prokop *et al.*, 1996) as well as maquette peptides (Robertson *et al.*, 1994), the latter complementing the characterization at the air/water interface (Strzalka *et al.*, 2000) after transfer to Si wafers incorporating MBE grown Ge–Si superstructures. From the deduced SLD profiles it is concluded that the helical bundles incorporated within the maquettes extend normal to the solid state surface (Strzalka *et al.*, 2001), even in cases when they were oriented parallel to aqueous surface prior to the transfer (Strzalka *et al.*, 2000). This is taken as evidence that substantial molecular rearrangement may occur upon transfer of even carefully engineered surface structures.

## 2. HBM's as synthetic membrane model mimics

A mongrel approach to mimicking lipid membrane surfaces by a solid supported system has been taken in Anne Plant's lab at the NIST by developing hybrid bilayer membranes (HBM's). In this approach, an alkanethiol SAM is prepared on a flat Au substrate, followed by the transfer of a (phospho-) lipid monolayer from the air/water interface (Plant, 1993). After drainage of surplus lipid material with filter paper, the resulting dual layer structure is stable even in ambient environment where the hydrophilic lipid headgroup is exposed to the gas phase. The molecular-scale structure of such systems has been investigated by a host of characterization methods (Meuse *et al.*, 1998), including neutron reflectometry (Majkrzak *et al.*, 2000). In very recent work it has then been demonstrated by studying the association of mellitin with HBM's that such systems provide a promising and versatile tool for the investigation of peptide/lipid interactions (Krueger *et al.*, 2001). At the same time, this work provides a reference for both the state-of-the-art of technical performance in neutron reflectometry measurements and sensible and realistic data evaluation. An example for the structure of the parent HBM system, as it emerges in terms of a neutron SLD distribution in an isotopically well-designed system (hydrogenated SAM, chain-perdeuterated lipid in the transferred monolayer adjacent to D<sub>2</sub>O) is shown in Fig. 8.

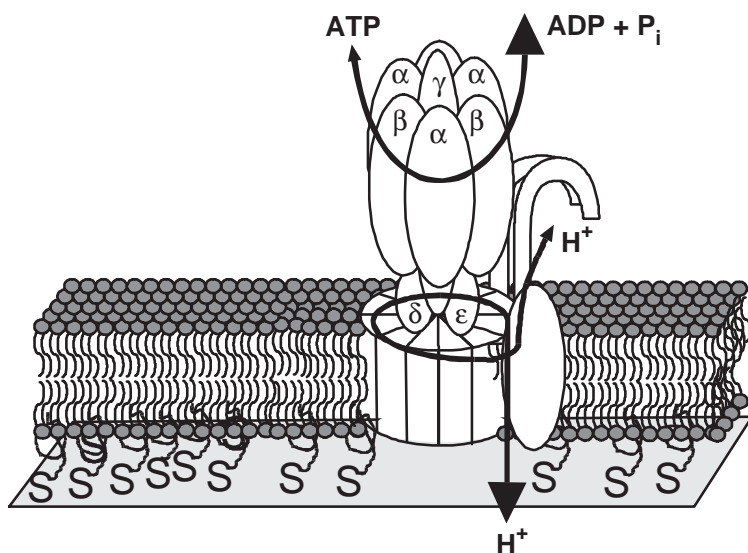


**Figure 8:** Structure of an octadecanethiol/DMPC HBM on a planar Au substrate as derived from neutron reflectometry. Displayed in the main figure is a family of neutron SLD profiles describing the structure of a HBM, composed of octadecanethiol and chain-perdeuterated DMPC, in contact with D<sub>2</sub>O. Each SLD profile is consistent with the experimental data shown in the inset. From Krueger *et al.* (2001) with permission

### 3. Tethered functional lipid bilayers

Solid-supported but simultaneously from the solid substrate spatially decoupled continuous lipid bilayers (Sackmann, 1996) are biophysicist's dream of a perfect mimic of membrane functions and at the same time synthetic chemist's and structural experimentalist's nightmare to synthesize and characterize in molecular terms. While Langmuir monolayers, SAM's, or HBM's are all quite obviously inadequate systems for the incorporation of large membrane proteins, even bilayers immediately adsorbed to solid substrates, such as the ones referred to above (Johnson *et al.*, 1991a, Koenig *et al.*, 1996, Charitat *et al.*, 1999), are clearly inadequate for the implementation of membrane functions, since the molecularly thin water layer that persists between the proximal bilayer leaflet and the solid substrate – 5 to 10 Å in thickness – is clearly insufficient to warrant the high lateral mobility of the lipid components within the structure required to retain most membrane functions and even more so to accommodate extramembrane sections that most membrane proteins incorporate. Thus, various concepts to incorporate tethering layers in between the solid substrate and the membrane have been put forward, *e.g.*, in the form of soft (polymer) cushions required to attach the membrane to the substrate by establishing covalent links between the solid and some of the lipid molecules while creating a stable and well-defined hydration layer of a thickness of some 10 to 100 Å between substrate and membrane (Knoll *et al.*, 2000), *c.f.* Fig. 9. Successful attempts on such schemes have attracted considerable attention (Cornell *et al.*, 1997, Raguse *et al.*, 1998).

Surface-sensitive scattering methods, and specifically neutron reflectometry, are particularly well suited to study the nanometer-scale structure of tethered lipid bilayer systems and their precursors. The adsorption of polyelectrolytes – such as poly(styrenesulfonate) – at the water surface (Saville *et al.*, 1994, Yim *et al.*, 2000), the molecular-scale conformation of hydrophilic polymer brushes at the air/water interface (Majewski *et al.*, 1997, Kuhl *et al.*, 1999, Politsch *et al.*, 2000, Wurlitzer *et al.*, 2000) as well as structured polyelectrolyte films deposited at wafer surfaces (Schmitt *et al.*, 1993, Lösche *et al.*, 1998, Steitz *et al.*, 2000) have thus been extensively studied. Polymer-supported monolayers in air and bilayers under water have been shown to form on adsorbed polyelectrolyte cushions (Wong *et al.*, 1999a) and have been characterized with respect to their molecular interactions with juxtaposed bilayers (Wong *et al.*, 1999b) where membrane fusion has been shown to occur. Synthetic routes for the formation of crosslinked polymer cushions have been



**Figure 9:** Schematic representation of a ATPase-functionalized polymer-tethered lipid bilayer. From Knoll *et al.* (2000) with permission

described (Seitz *et al.*, 2000). Alternate promising strategies for the formation of stabilized (Küpcü *et al.*, 1995, Schuster *et al.*, 1998b) bilayer membranes involve S-layers as carrier materials (Wetzer *et al.*, 1997). The incorporation of membrane proteins in such systems has already been demonstrated (Schuster *et al.*, 1998a) and the lateral mobility of the constituent phospholipids has been characterized

via photobleaching (FRAP) measurements (Györvary *et al.*, 1999, Starr and Thompson, 2000). For a deeper understanding of the self-assembly process that leads to the formation of S-layer-supported membranes a molecular-scale comprehension of the lipid/peptide interface, as derived from surface-sensitive scattering methods (Weygand *et al.*, 1999, Weygand *et al.*, 2000), has been proven invaluable. The future holds great promises and great challenges in this rapidly developing field. Surface-sensitive X-ray and neutron scattering methods will be one of the primary tools that drive this development.

## ACKNOWLEDGMENTS

A warm *thank you* to J. Kent Blasie, Giovanna Fragneto-Cusani, Ka-Yee C. Lee, Tim Salditt, Markus Weygand, and Steve H. White for fruitful discussions, for making available preprints and/or for the communication of unpublished work. I thank Kristian Kjaer for his devising of the liquid surface experimental station at BW1 and valuable advise for the experimental work, as well as Markus Weygand and Peter Krüger for measurements. I am very grateful to U. B. Sleytr, B. Wetzer, and D. Pum for a long-standing and fruitful collaboration in the field of S-layer characterization. Beam time at HASYLAB (DESY, Hamburg, Germany) under project no. II-99-078 is gratefully acknowledged. The work has been financially supported by the DFG through the SFB 294 (TP F3) and by the Fonds der Chemischen Industrie (Frankfurt, Germany).

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