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REVIEW

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Use of X-ray scattering to aid the design and delivery of membrane-active drugs

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Abstract Biological membranes can be targets for compounds that either disrupt their barrier function or affect protein function via membrane-mediated processes. Biophysical studies on membrane-mimetic systems composed of membrane lipids have contributed substantially to our knowledge on the pertaining membrane physics and aid the development of membrane-specific drugs. Moreover, lipid membranes and, in particular, liposomes are convenient systems for drug delivery. We review some of our recent work that demonstrates the applicability of X-ray scattering to understanding the molecular mechanisms of drug–membrane interactions. In particular we focus on effects of anesthetics, sphingomyelinase, and antimicrobial peptides. We further discuss X-ray scattering as a quality-control tool for liposomal drug-delivery systems.

Keywords Lipid bilayer · Non-lamellar phases · Micelles · Liposomes · Lipid–drug interactions · Lipid–protein interactions

Introduction

Biological membranes are one of nature's most fascinating materials, designed to separate the inner life of cells or organelles from the "outer world". Shielding or compartmentalization has been an important evolutionary step, yet biological membranes also need to exchange materials or communicate with surrounding cells and they do so in many different ways. Further, membranes provide important substrates for chemical reactions, thus establishing biological membranes as the central bio-interface of life. Their functional layer is a composite of lipids and proteins. First realistic models of this layer considered the proteins to be dissolved in a fluid matrix built up by the lipids (Singer and Nicolson 1972). Newer models picture typical membranes as crowded, and of different thickness, with molecules distributed non-randomly (Engelman 2005), leading to the formation of functional domains, for example membrane rafts (Lingwood and Simons 2010).

Envisaging membranes as central bio-interfaces of life almost naturally leads to concepts that put biological membranes in the focus of research attempting to use them as targets for medical drugs (Escriba et al. 2008). However, specific interactions of drugs with membrane-bound proteins or receptors are not the purpose of these efforts. Rather, membrane lipids and their collective properties are in the focus of research conducted with the purpose of physically interfering with their function as a barrier and/or regulator of protein function. The reasons are obvious. First, it is a property of lipids to form a membrane, not that of proteins. Second, even if the total mass of proteins in membranes is large (on the order of 60 %), the proportion of proteins bounded by the membrane is only 10–15 % (Heberle and Feigenson 2011), the rest is located in the extracellular or intracellular space. Thus, functional properties of biological membranes will be largely affected by those of its constituent membrane lipids, a fact that also became a research topic with the emergence of lipidomics (Mouritsen 2005).

However, any commercial drug must act specifically. Specificity is easily seen in so-called key–lock mechanisms,

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in which drugs target a specific protein sequence or structure. The specificity of membrane lipids arises from the specific distribution of lipids in cell membranes. It is well known that the lipid composition of biological membranes differs substantially between different cell types. This knowledge can be used, for example, in the design of compounds to treat bacterial infections (Lohner 2001). Eukaryotic plasma membranes are characterized by asymmetric distribution of phospholipids between the outer and the inner lipid leaflet of the bilayer, with phosphatidylserine (PS) and phosphatidylethanolamine (PE) on the cytoplasmic side of the membrane, whereas phosphatidylcholine (PC) and sphingomyelin (SM), occur predominantly in the external leaflet of the bilayer (van Meer et al. 2008; Lohner et al. 2008). Additionally, eukaryotic plasma membranes contain substantial amounts of cholesterol (25–50 mol%); a lipid which is absent in prokaryotic cells. More importantly, prokaryotes expose anionic lipids, for example phosphatidylglycerol (PG) or cardiolipin (CL), to the extracellular side (Lohner et al. 2008) making them targets for cationic host defense peptides (Lohner and Blondelle 2005), although hydrophobic interactions are also important. A somehow related concept is currently being investigated for treatment of cancerous cells (Riedl et al. 2011b). Here, the asymmetric lipid architecture of eukaryotic plasma membranes is abolished leading to accumulation of negatively charged PS in the outer membrane leaflet of the cancerous cells (Riedl et al. 2011a).

In addition to the design of membrane-active drugs their transport and release at a specific site of interaction is of equal importance. Here, ever since the pioneering work of Bangham et al. in the 1960s and 1970s, liposomes with a well-defined lipid composition have attracted much attention. In general, liposomes have many attractive biological properties for medical purposes, because they are biocompatible, biodegradable, and, in principle, of low toxicity and low immunogenicity. Primarily, liposomes were developed as drug-delivery systems with the objective of improving the therapy of diseases as diverse as cancer, infections, or cardiovascular pathologies. To date, several of these liposomal products have been approved for clinical use, or are at the final stage of clinical development. Some products are already commercially available (Torchilin 2005; Wagner et al. 2006). Recent developments focus on liposomes as smart delivery systems for controlled release and specific cellular targeting, serving as essential building-blocks for nano-medicine (Torchilin 2006; Musacchio and Torchilin 2011). Apart from therapy, liposomes are being investigated for imaging and diagnosis with the objective of using them as multifunctional nanoparticles for contrast-enhanced molecular diagnostics. Taken one step further, liposomes are regarded as theranostics, combining both therapy and diagnostics (Lammers et al. 2011; Kunjachan et al. 2012). However, to meet all these expectations a plethora of basic research work is required to ensure the highest quality for pharmaceutical products. Pharmaceutical liposomes must fulfill several basic requirements, for example reproducible fabrication, high stability, and structural integrity, while maintaining the activity of the therapeutic contents. Having this in mind, it is of central importance to conduct thorough biophysical characterization before any pre-clinical testing.

Clearly, no single technique in this field is capable of addressing all issues related to the design of novel membrane-active compounds and liposomal drug-delivery systems. Thus, in truth, a combination of several methods with different experimental windows in time and space are required. However, X-ray scattering experiments are particularly important because they offer non-invasive and label-free insight into global membrane properties. For example, X-rays do not reveal the local interaction of drugs with membrane lipids in their immediate environment. They reveal the effects of the drugs on the membrane at a global level, i.e. bulk properties, for example structure and elasticity. These bulk properties, however, determine membrane integrity and/or membrane-mediated effects on proteins that insert in or are associated with the membrane. Thus, membranes are used as sensors to monitor the effects induced by the compounds. This article reviews some recent achievements in this context.

What to expect from X-ray experiments

X-rays are, in general, sensitive to positional correlations, whose origin may be either short-range or long-range (Chaikin and Lubensky 1995). In membrane-mimetic systems, both types of correlation can be found. Fluid bilayers, for example, are liquid-like in the plane of the bilayer, where the acyl chains exhibit short-range order (Spaar and Salditt 2003). Membrane bilayers often form stacks, either as multilamellar vesicles (MLVs), or as highly aligned multibilayers on solid substrates (Salditt 2005; Pabst et al. 2010). This positional order is long-range, but not quite. Because of bending fluctuations of fluid membranes, the positional order is rendered to long-range (de Jeu et al. 2003; Pabst et al. 2003). This has an important consequence for the experimental window of X-ray scattering on membrane assemblies. It gives the Bragg peaks a particular cusp-like shape described by the Caillé theory (Zhang et al. 1994). Thus, detailed analysis of peak shapes, gives information about bending fluctuations and, hence, membrane elasticity (Pabst et al. 2000). Another frequent case is that the membranes, because of steric or electrostatic repulsion, form unilamellar vesicles (ULVs). ULVs have no positional order normal to the plane of the bilayer. Then,

the small-angle X-ray scattering (SAXS) regime does not exhibit any peaks but a diffuse modulation of scattered intensity, because of the electron density distribution along the lipid bilayer. A detailed review of the analysis of X-ray data of model membranes has been given elsewhere (Pabst et al. 2010; Salditt 2005; Rappolt and Pabst 2008; Rappolt 2006) (see also the article by Heberle et al. in this issue). Here, it suffices to name the most important parameters that can be derived from these experiments.

One of these parameters is the membrane thickness, d_B, which is often defined either as the headgroup-to-headgroup distance, the Luzzati thickness, or the steric bilayer thickness (Kucerka et al. 2008; Rappolt and Pabst 2008). Another central parameter for membrane structure is the lateral area per lipid, A. It describes the lipid packing density and is also a key parameter to guide molecular dynamics simulations (Pabst et al. 2010). Both structural parameters are sensors for the membrane effects of compounds that lead to corresponding changes in membrane structure and can be determined directly from diffraction experiments.

Besides loss of membrane integrity, going in hand with a loss of its barrier function insertion of compounds may have secondary effects on protein function (Cantor 1997; Dan and Safran 1998; Killian 2003). In particular, they may affect the distribution of lateral pressures in the membrane, such that conformational equilibrium of membrane proteins is affected (Marsh 2007). These membrane-mediated effects are determined by elastic parameters of the lipid monolayers, for example the bending rigidity κ_m, spontaneous curvature c_0 and Gaussian curvature modulus, κ_G. To measure these parameters X-ray scattering experiments have to be performed under osmotic stress (Pabst et al. 2010). This enables determination of bilayer bending rigidity $K_C = 2\ \kappa_m$. Alternatively, K_C can be determined by use of X-ray surface diffraction techniques (Lyatskaya et al. 2001; Liu and Nagle 2004; Salditt 2005; Pan et al. 2008) on highly-aligned lipid multibilayers without the need to apply osmotic stress.

Spontaneous curvatures can be determined in inverted hexagonal phases. Those lipids which do not form inverted hexagonal phases can be transferred into hexagonal phases by use of lipid templates (Rand et al. 1990; Chen and Rand 1997; Leikin et al. 1996; Vacklin et al. 2000; Alley et al. 2008). Further, κ_m can be determined in hexagonal phases by use of osmotic stress (Rand et al. 1990; Rappolt and Pabst 2008). Finally, the Gaussian curvature modulus can be estimated from κ_m (Marsh 2007) or determined by use of cubic template phases (Siegel 2008).

Another important issue concerns phase separation in lipid mixtures and the formation of lipid domains, which are considered as simple models for membrane rafts. Given that the formed lipid domains are stable and in registry, X-ray scattering can be used to detect those domains (Staneva et al. 2008; Karmakar et al. 2006; Staneva et al. 2009; Pabst et al. 2009; Boulgaropoulos et al. 2010; Uppamoochikkal et al. 2010; Boulgaropoulos et al. 2011) and to retrieve the same parameters listed above, although literature is scarce in this respect. Submicron-sized lipid domains have also been characterized by neutron scattering using either the possibility of contrast variation (Gliss et al. 1998; Pencer et al. 2005) or via variation of the neutron coherence length (Armstrong et al. 2012). Recently, Nagle and coworkers demonstrated the use of wide-angle X-ray scattering on highly aligned multibilayers to detect and analyze domains which are not in registry (Mills et al. 2008a, b).

In summary, X-ray scattering reports on global membrane structure and elasticity. With regard to the effects of membrane-active drugs these data can be used to detect how these compounds change the bulk properties of membranes directly and/or how these changes may affect the functioning of membrane proteins as an indirect effect. In the following text we will present few selected recent examples to illustrate these points.

Lipid–drug interactions

Drugs that target membranes may have various specific effects on the lipid bilayers, depending on the physicochemical properties of the compounds and the membrane lipids. In order to generalize these effects it is advantageous to consider the lateral pressure profile of lipid membranes, which describes the variation of repulsive and attractive interactions across the bilayer (Ben Shaul 1995). For example headgroup and hydrocarbon chain interactions are repulsive in nature, while minimizing the exposure of the hydrophobic core to the aqueous phase leads to attractive pressures at the apolar/polar interface of the bilayer. For ideally flat, stable, bilayers the net sum of attractive and repulsive lateral pressures must be balanced. Then in respect of lipid–drug interaction four different scenarios can be conceived:

1. the compound adsorbs to, or inserts into, the membrane without leading to changes in the lateral pressure profile;
2. the compound inserts and modifies the lateral pressure profile, but does not perturb the membrane integrity;
3. compound insertion leads to an overall structural change, for example the formation of an inverted hexagonal or cubic structure; and
4. the interaction of the drug with the membrane leads to partial disintegration of the membrane, e.g. pore formation, or to its complete lysis (detergent-like effect).

Case 1 is desired for liposomal drug-delivery systems. Case 2 can lead to indirect, membrane-mediated effects on the functioning of membrane proteins, and cases 3 and 4 irreversibly destroy membrane barrier function. For different medical applications all these scenarios apply and the drugs have to be adjusted carefully to one of these categories. In the following discussion we will give some examples of indirect and direct effects, with emphasis on the contributions from X-ray scattering.

A variant of the lateral pressure concept that is frequently discussed in the literature is hydrophobic matching (Mouritsen and Bloom 1984; Killian et al. 1996; Killian 2003), i.e. the hydrophobic transmembrane domain of proteins need to be matched to the thickness of the hydrophobic core of the lipid bilayer. This may lead to local distortions of the bilayer in the vicinity of the inserted protein or to tilting or conformational changes of the transmembrane helices or even protein aggregation (Killian 2003).

Indirect effects

Channel proteins

Ion channels enable selective transport across the membrane and may be gated by ligand-binding, voltage changes, or mechanically by membrane tension. Irrespective of the gating mechanism, these proteins need to open up a selective pore, which causes lateral expansion of the protein. It is not yet possible to state firmly that lateral expansion generally occurs with channel opening, because of the limited availability of crystallographic data. Examples, however, where lateral expansion has been found are a ligand-gated ion-channel (Hilf and Dutzler 2008, 2009) and mechano-sensitive channels (Chang et al. 1998; Yefimov et al. 2008). Changes in the cross-sectional area of channel proteins require work that must be performed against the lateral pressure profile $p(z)$ (Fig. 1):

$$W = -\int \Delta A_p(z) p(z) dz,$$

which is the two-dimensional analog of the work that must be performed to compress a given volume, known from thermodynamics. The change in the lateral area of the protein is associated with a transition from a closed to open state $r \rightarrow t$. In equilibrium the distribution of closed versus open states can be described by the equilibrium constant:

$$K_0 = \frac{[r]_0}{[t]_0}.$$

A compound that inserts into the membrane, or in general changes in membrane architecture caused by external factors, for example stress, enzymes, etc., leads to a change of the lateral pressure profile $[p(z) \rightarrow \tilde{p}(z)]$. Hence, the work to open the channel—now against $\tilde{p}(z)$—is modified and, consequently, so is the distribution of open to closed channels. The new equilibrium is directly related to the initial one (Cantor 1997)

$$K = \frac{[r]}{[t]} = K_0 e^{\alpha} \text{ with } \alpha = \frac{1}{k_B T} \int \Delta A_p(z) \Delta p(z) dz\,.$$

Thus, if the change in work to open the channel is positive ($\alpha > 0$), then the distribution of open states decreases and vice versa for $\alpha < 0$.

Hence, changes in the conformational equilibrium depend on changes in the cross-sectional area associated with the opening of the ion-channel and the shifts in lateral pressure profile because of insertion of the membrane-active compound. The largest changes in the lateral pressure profile occur at the polar/apolar interface, i.e. close to the glycerol backbone in the case of glycerophospholipids (Fig. 1). Hence, the largest modifications in the distribution of lateral pressures can be achieved by compounds that reside in this interfacial area, for example general anesthetics.

Anesthetics have a long and very successful history in surgery. However, their molecular mode of interaction is still unknown. Anesthetics might either act directly on post-synaptic ion channels or indirectly via a membrane-mediated mechanism. With focus on the latter mechanism, effects of ketamine on the pressure profile have been reported recently (Jerabek et al. 2010). Lipid bilayers composed of palmitoyl oleoyl phosphatidylcholine (POPC) served as simple models for nerve membranes. Pressure profiles were derived by a combination of SAXS and molecular dynamics simulations. Ketamine was found to insert, predominantly, close to the polar/apolar interface, causing a net shift of lateral pressures toward the center of the lipid membrane. These results were then projected on the opening probability of ion-channels, which were mimicked by using simple geometric models (Fig. 2). It was found that approximately 2 mol% ketamine is sufficient to inhibit half of a channel composed of bent helices, whereas it was necessary to use 18 mol% ketamine to inhibit 50 % of a channel composed of tilted helices. These results demonstrated:

1. that general anesthetics indeed may affect membrane protein function via a membrane-mediated mechanism; and
2. that the lateral pressure mechanism gives a realistic explanation of effects observed by electrophysiology.

Surface-active proteins

Application of membrane-active drugs may interfere with specific cellular pathways and activate signaling cascades.

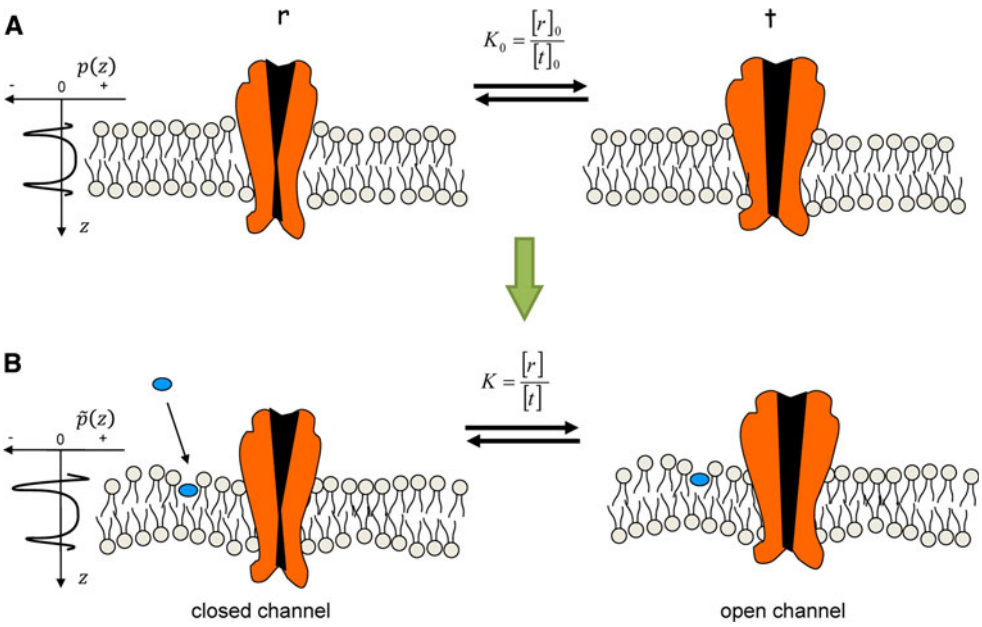

Fig. 1 Mechanical coupling of bulk membrane properties to the conformational equilibrium of ion channels. The initial equilibrium is characterized by opening of the central pore of the channel protein (change from state *r* to *t*) requiring lateral expansion of the protein against the lateral pressure profile $p(z)$ (**a**). If a compound inserts into the membrane the lateral pressure profile may be changed, leading to a new conformational equilibrium of the pore protein (**b**). This will be most effective if the compound inserts close to the polar/apolar interface, where changes of $p(z)$ are largest

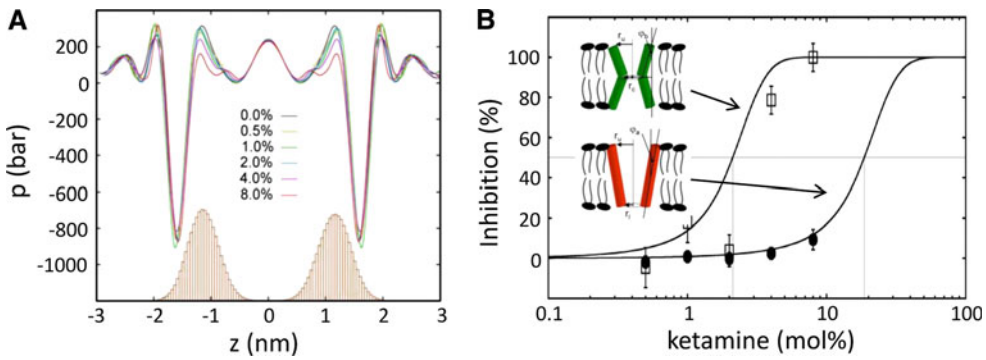

Fig. 2 Lateral pressure profile of POPC bilayers in the presence of ketamine (**a**). Ketamine inserts close to the polar/apolar interface (*bars* represent ketamine distribution function) and leads to shifts of the profile toward the membrane interior. *Numbers* indicate ketamine concentration in mol%. **b** Theoretical inhibition of ion channels with opening mechanisms that involve either bending or tilting of helices (*inset*). *Gray lines* indicate the IC_{50} values, which are 2 mol% for bent helices and 18 mol% for tilted helices. Data taken from Jerabek et al. (2010)

It is therefore also of interest to study the effects of compounds or proteins on plasma membranes that are released or activated during such processes. For example, sphingomyelinase (SMase), is one of the central enzymes that is activated during the execution of apoptosis, but is also associated with other physiological processes, for example lipid oxidation (van Blitterswijk et al. 2003; Fruhwirth et al. 2007). SMase is a lipase that hydrolyzes sphingomyelin (SM) to ceramide. Ceramide, generated this way, has properties which are distinct from SM. Here, the focus is on long-chain (palmitoyl) ceramide. In particular, the cone-like molecular shape of ceramide, smaller lateral area per lipid, and their high melting temperature are properties which cause significant changes in lateral membrane structure (Shah et al. 1995; Veiga et al. 1999).

If hydrolysis of SM by SMase proceeds only in one of the lipid monolayers, the ceramide-containing leaflet will shrink laterally because of the smaller lipid area of ceramide, causing a budding effect very similar to observations of apoptotic body formations (Holopainen et al. 2000a). A symmetric distribution of ceramide in turn has been demonstrated to cause a macroscopic phase separation into fluid (L_α) and gel (L_β) domains (Holopainen et al. 2000b; Goni and Alonso 2006; Fidorra et al. 2006; Castro et al. 2007; Staneva et al. 2008; Chiantia et al. 2008; Pabst et al. 2009; Boulgaropoulos et al. 2010). This implies that ceramide causes lipid sorting. In particular, it has been shown that ceramide predominantly interacts with SM, for example via hydrogen bonding, and that both lipids are enriched in the L_β domains (Boulgaropoulos et al. 2011). Detecting phase separation, in general, is a challenge to any

technique as it involves a coupling of the experimental window of the method (time and length scale) to the size and stability of the lipid domains. Thus, domains can only be detected if the experimental window is appropriate. On the other hand, compositional fluctuations (unstable domains) cannot be differentiated from stable domains, if the time and length scales of the applied technique are shorter than domain lifetime and size, respectively. Consequently, a combination of experimental techniques with different experimental windows is mandatory. For example, we recently combined X-ray scattering with infrared spectroscopy and calorimetry to differentiate stable from unstable lipid domains in mixtures of SM, ceramide, and POPC (Boulgaropoulos et al. 2011). The pseudo-binary phase diagram that was constructed this way demonstrates that domains near the phase transitions are not stable, but correspond to compositional fluctuations. Distant from these transitions, however, the domains are stable. Such information can help to reconcile discrepancies in compositional phase diagrams because of application of a single experimental technique only (Marsh 2009, 2010).

Additionally, when discussing lipid-mediated effects on proteins in domains, knowledge on domain stability is necessary. For stable lipid domains, insight can be achieved by combining X-ray scattering with osmotic stress (Pabst et al. 2010). This concerns, in particular, the thickness and the bending rigidity of the coexisting domains. The membrane thickness, in general, can be derived either by a Fourier synthesis, using the intensities of the Bragg reflections, or by using a global, full q-range, model (Pabst 2006). The number of Bragg peaks in the L_α phase is usually too small to derive reliable electron density profiles and global analysis techniques are better suited in these cases (Pabst et al. 2000). However, no global analysis technique has been developed for coexisting fluid or fluid-gel domains. Application of osmotic pressure reduces bilayer bending fluctuations and, hence, increases the number of observed Bragg peaks. This enables one to calculate the electron density profiles and hence the membrane thicknesses of the coexisting phases, as demonstrated, for example, for the SM–ceramide–POPC mixture (Pabst et al. 2009). Additionally, by variation of osmotic pressure, one can measure the isotherms (Fig. 3) to probe the forces between like domains in a membrane stack (Pabst et al. 2009). Steric repulsion because of bending fluctuations, hydration repulsion, and van der Waals attraction have been probed. One of the central parameters determined by this analysis is the bending rigidity of the fluid domains. This information has been used to estimate the effects of lipid sorting on the opening of ion-channel proteins as a function of channel size. Significant changes were predicted (Pabst et al. 2009). Most recently, we performed a similar study of SM–ceramide and POPC, but in the presence of cholesterol, focusing on the competition between cholesterol and ceramide for SM (Boulgaropoulos et al. 2012). In addition to the previously published report we also determined the spontaneous curvatures of the domains from X-ray experiments, which enabled us to obtain better estimates for the effects on ion channels. Results showed that cholesterol helps to stabilize the function of membrane channels in fluid domains.

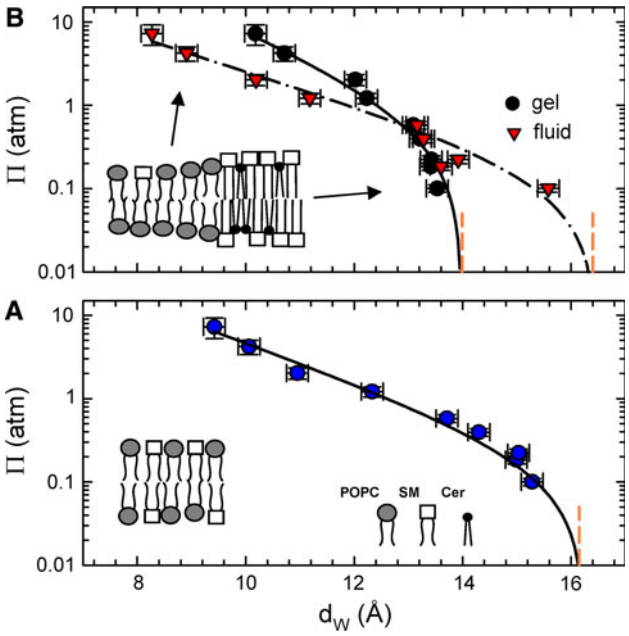

Fig. 3 Osmotic pressure measurements of interacting forces in phase separated lipid membranes. Osmotic pressures, Π, are adjusted by variation of the poly(ethylene glycol) concentration and the bilayer separation d_W is determined from X-ray measurements. **a** The equation of state for a homogenous mixture of POPC and SM. **b** The isotherms for coexisting L_α and L_β phases for a mixture of POPC–SM and ceramide (*Cer*)

Thus, X-rays are valuable tools to study the properties of membrane domains and the restructuring effects on membrane proteins. Additionally, with the aid of high photon flux at synchrotron beamlines time-resolved studies on the activity of SMase were performed (Boulgaropoulos et al. 2010). For this purpose unilamellar SM/POPC vesicles were prepared by extrusion and rapidly mixed with a solution containing SMase and exposed to X-rays with a time resolution of 1 min. The study showed that L_β domain formation proceeds about four times faster than hydrolysis of SM, because of preferential pairing of ceramide with SM (Fig. 4). Interestingly, studies correlated with in-vitro studies of SMase activity with regard to SM hydrolysis, suggesting that the enzyme is regulated by membrane properties.

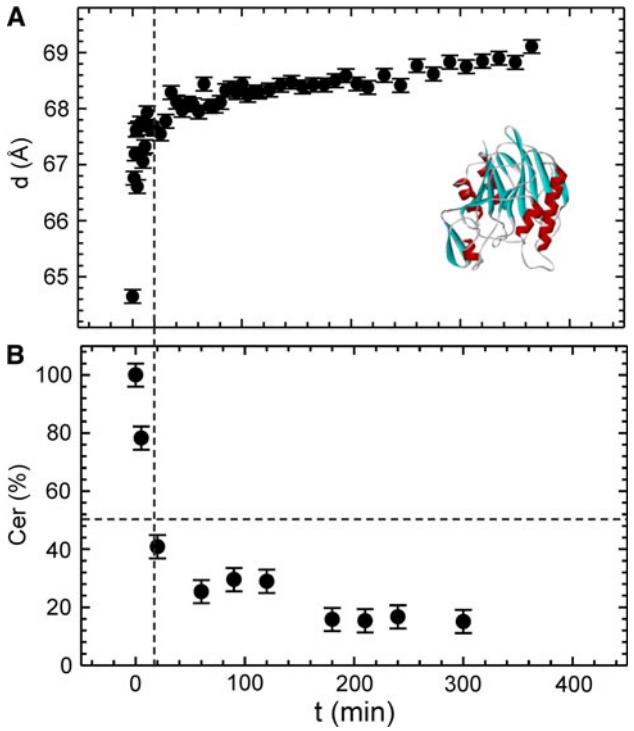

Fig. 4 Coupling of enzyme activity to bulk membrane properties as revealed by synchrotron time resolved X-ray scattering (Boulgaropoulos et al. 2010). The structure of SMase is shown in the *inset*

Direct effects

As outlined above, membrane-active drugs can have quite diverse effects on global bilayer structure. They can induce thickness changes, partial disintegration (pore formation), or micellization (detergent-like effect). Such effects can be monitored directly by X-ray techniques, as described below for antimicrobial peptides as examples.

Effects on global membrane structure

Bacterial cell membranes of, e.g., *Escherichia coli* or *Acholeplasma laidlawii* contain substantial amounts of so-called non-lamellar phase-forming lipids, for example PE, CL, or monoglucosyldiacylglycerol (McElhaney 1992; Rilfors et al. 1993; Morein et al. 1996). In terms of the lateral hydrocarbon chain pressure profile these lipids lead to an increase of the lateral pressure in the center of the bilayer (curvature frustration). Non-lamellar structures of these lipids are primarily inverted hexagonal (H_{II}) but also cubic (Siegel and Banschbach 1990; Rappolt et al. 2003). Other major membrane lipid components, for example PG or PC are of cylindrical shape and hence form bilayer structures (Seddon and Templer 1995). A balance of bilayer and non-bilayer-forming lipids is believed to be fundamental to the functioning of membranes (Gruner 1985; Cantor 1999).

It has been proposed that antimicrobial peptides may tip the balance of bilayer versus non-bilayer-forming forces toward non-lamellar structures, leading to membrane disintegration for bacteria with high content of non-lamellar phase forming lipids (Lohner and Blondelle 2005). The extent of membrane perturbation depends on a number of parameters, for example concentration, molecular size, and aggregation state of the peptide, and on the membrane penetration depth of the peptide. Hence, there is tight coupling between the properties of the peptide and those of the lipid bilayer.

A correlation between antimicrobial activity and non-lamellar phase formation of *E. coli* lipid extracts was recently reported for cationic amphipathic peptides derived from a fragment of human lactoferrin (Zweytick et al. 2008, 2011). X-ray experiments revealed the coexistence of two bicontinuous cubic phases belonging to space groups Pn3m and Im3m. The lattice spacing of both cubic phases was strongly reduced in the presence of these peptides, which was explained by an increase of spontaneous curvature resulting from a reduction of the effective lipid head group area because of electrostatic interactions between lipids and peptides. Furthermore, promotion of cubic phases of lipid extracts from *E. coli* or *A. laidlawii* was also reported for gramicidin S, a cyclic antimicrobial peptide (Staudegger et al. 2000). It was proposed that its capacity to strongly promote negative curvature and thus cubic phase formation was because of the limited flexibility of the β-turn of gramicidin S and the clustered location of the ornithine side chains.

In turn, cytolytic melittin stabilized the bilayer structure of mixed chain PE (Hickel et al. 2008), whereas the antimicrobial peptides nisin (El Jastimi et al. 1999) and a synthetic 17 β-amino acid oligomer (Epand et al. 2003) stabilized the inverse hexagonal phase of PEs. Both peptides promote negative curvature and hence formation of H_{II} structures—nisin because of insertion of its large hydrophobic section (aa 1–19), causing an increase of the hydrophobic volume in the bilayer interior, and the 17 β-amino acid oligomer as a result of expanding the regions of the bilayer below the pivotal plane more than regions close to the interface. Furthermore, coexisting bilayer and cubic structures were observed for the antimicrobial α-helical PGLa and the β-sheet protegrin-1, whereas gramicidin S induced a complete transition from bilayer to cubic structures (Hickel et al. 2008) (Fig. 5). Alamethicin also promoted cubic phase formation, when incorporated in small amounts in dielaidoyl-PE (Keller et al. 1996), it was suggested this was because of changes in the thickness and/or flexibility of the lipid bilayer.

Membrane thinning has been observed in a number of lipid–peptide studies and is frequently associated with pore formation (see below). At low peptide concentrations

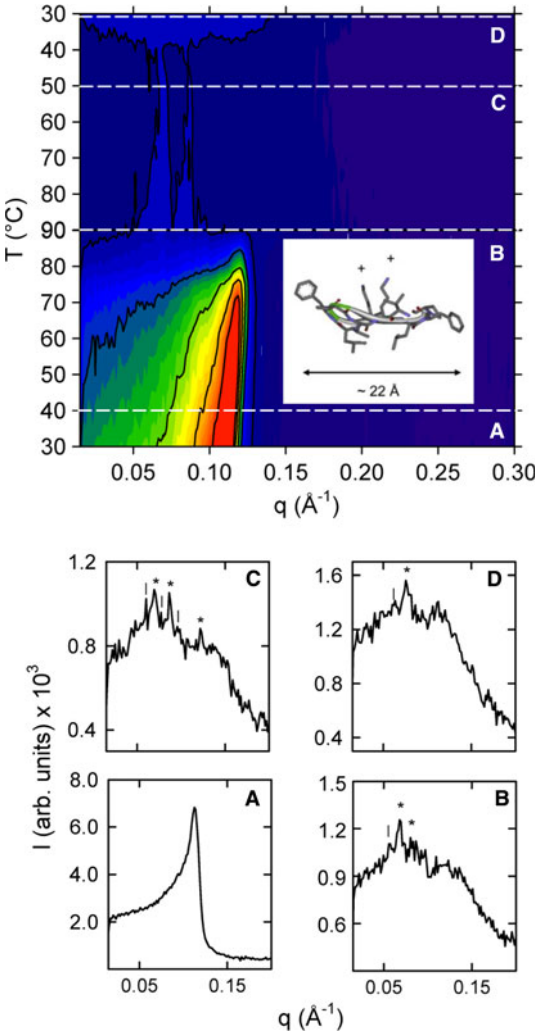

Fig. 5 Phase behavior of POPE containing gramicidin S (structure shown in *inset*) as determined from X-ray scattering (Hickel et al. 2008). The *upper panel* shows a contour plot of SAXD patterns. The *lower panel* shows selected diffraction patterns at 40 °C (**a**), 90 °C (**b**), 50 °C (**c**, cooling) and 30 °C (**d**, cooling). At 90 °C and below, upon cooling, the system has two cubic phases Pn3m (*asterisk*) and Im3m (*vertical line*)

amphipathic peptides are adsorbed by the membrane with its helical axis parallel to the membrane surface occupying space in the headgroup region of the lipids (Lohner and Blondelle 2005; Huang 2006). Thereby they induce voids in the hydrophobic core of the bilayer, which, to some extent, can be compensated by increased disorder of the lipids' acyl chains and/or by moving the opposite leaflet toward the hydrophobic face of the peptides (Lohner 2009). As a consequence dimples will be created around the peptide site (Fig. 6a) and overall a membrane thinning can be observed, as verified from the strongly reduced membrane thickness calculated from small-angle X-ray data (see below). In its extreme case, and in particular for rigid membranes, i.e. in the gel phase, peptide insertion can result in the formation of quasi-interdigitated structures (Sevcsik et al. 2007, 2008) (Fig. 6b). LL-37 induced such a structure in negatively charged PGs. The presence of the quasi-interdigitated structure was most prominent for the longest chain PG (diC18:0) and was only detected to some extent for long chain PCs (diC20:0 PC). Very similar effects were also observed for melittin and PGLa, although differences in the ability and extent of the induction of a quasi-interdigitated structure were found that can be attributed predominantly to the different size of the peptides. With the help of X-ray data, it was also possible to show that these structures always coexist with a lamellar gel phase (Sevcsik et al. 2007, 2008).

Pore formation

Membrane thinning, as a result of local dimple formation at low peptide concentrations, and hydrophobic matching at high peptide concentrations has been associated with peptide pore formation (Huang 2006). Extensive X-ray diffraction and neutron scattering experiments combined with oriented circular dichroism measurements showed that below a critical concentration antimicrobial peptides, for example magainin 2, protegrin-1, melittin, or alamethicin, are aligned parallel to the bilayer plane (S-state or surface state), whereas above this concentration they are oriented perpendicularly to this plane (I-state or inserted state) (Huang et al. 2004). The transition from the S to the I-state has a sigmoidal peptide-concentration dependence indicating cooperativity in the peptide-membrane interactions. Two different types of peptide pores can be discerned. Barrel-stave pores are typically displayed by α-helical amphipathic peptides with low surface charge, for example alamethicin, whereas highly charged α-helical amphipathic peptides, for example melittin, usually form toroidal pores (Zemel et al. 2005). X-rays, in general are not sensitive to membrane pores. Here neutron scattering and the possibility for contrast variation was pivotal to measurement of the different pore sizes (He et al. 1995). X-rays, in turn, provide a global average of the structure and are therefore sensitive to membrane thinning. The thinning of the fluid bilayer, observed to be in the range 2–3 Å, is an average value over local dimple deformation (Huang 2006). X-ray scattering combined with osmotic stress experiments further demonstrated that pore formation by alamethicin reduces the bending rigidity of di-unsaturated PC bilayers (Pabst et al. 2007). If X-ray data are combined with MD simulations, also location and orientation of peptides in membranes can be determined, as demonstrated recently for alamethicin in PC membranes (Pan et al. 2009).

The same authors also showed a clear correlation between the hydrophobic length of the peptide and membrane thickness (Pan et al. 2009). Consequently, membrane

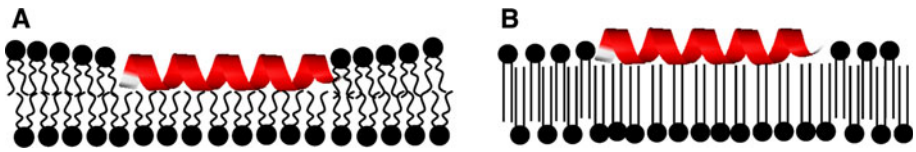

Fig. 6 Schematic diagrams of peptide-induced dimple formation because of elastic deformation of the membrane (**a**) and the quasi-interdigitated phase (**b**), which forms below the melting transition temperature in rigid bilayer structures

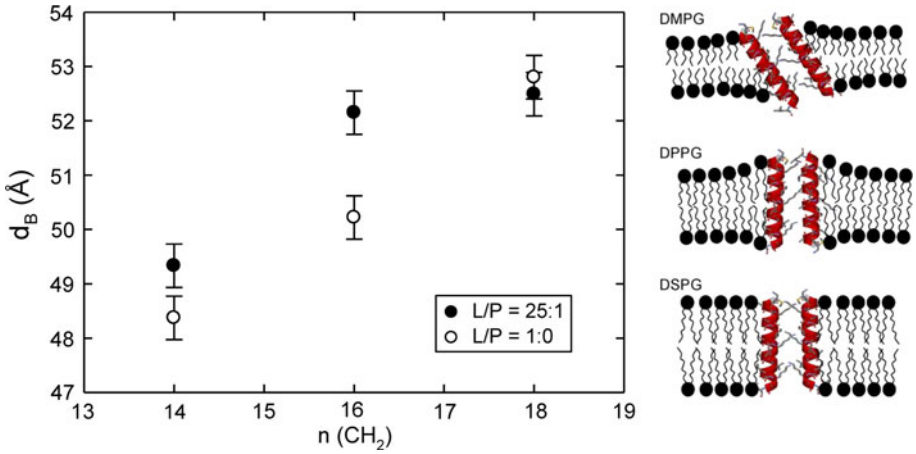

Fig. 7 Change of membrane thickness in fluid PG bilayers upon addition of PGLa as a function of chain length (Pabst et al. 2008). P/L is the peptide to lipid molar ratio. For DMPG and DPPG bilayers an increase of membrane thickness was observed in the presence of the peptide, whereas for DSPG membranes no significant change in membrane thickness was observed. Findings can be understood as an interplay of hydrophobic length of the peptide and the ability of the hydrophobic core to stretch in order to adjust to the peptide's length. For DMPG bilayers it is energetically more favorable to tilt the peptide helices within the membrane than to adjust the membrane core to helices inserted normal to the membrane surface. Schematic diagrams in the *right panel* depict the different scenarios

thinning need not necessarily precede pore formation, as shown for the antimicrobial peptide PGLa (Pabst et al. 2008). For distearoyl-PG (DSPG) no effect on the bilayer thickness was observed, because of matching of the hydrophobic length of the peptide with the hydrophobic core of the bilayer. On the other hand, PGLa caused membrane thickening for the shorter dimyristoyl-PG (DMPG) and dipalmitoyl-PG (DPPG), revealing the importance of hydrophobic matching and that membrane thinning is not a unique signal for peptide pore formation (Fig. 7).

Disk-like micelles with a peptide rim

Finally, there are findings indicating that antimicrobial peptides may damage the bacterial membrane via a detergent-like mode of action (Bechinger and Lohner 2006; Bechinger 2009). The character of, particularly, linear amphipathic α-helical peptides shows some analogies with detergent molecules. For example, melittin, with its overall amphipathic character composed of a cluster of cationic amino acids at the C-terminus and a stretch of hydrophobic amino acids, resembles the features of many detergents characterized by a polar/charged headgroup and a hydrophobic moiety. Indeed, at high melittin concentrations (lipid-to-peptide molar ratio 15:1) disk-shaped particles were found for a melittin–dimyristoyl–PC (DMPC) mixture (Dufourc et al. 1986; Monette and Lafleur 1995), suggesting detergent-like solubilization of the membrane under these experimental conditions. Gradual membrane disintegration was also observed in staphylococcal δ–lysin/DMPC aggregates (Lohner et al. 1999). Modeling of the diffuse SAXS data yielded disk-like micelles of approximate diameter 14 nm and bilayer thickness 5.2 nm surrounded by a peptide rim approximately 1 nm thick. The latter agrees well with the cross-sectional area of a peptide helix. Similar peptide–lipid aggregates with a diameter of 27 nm and a bilayer thickness of 5.5 nm were found in the gel-phase for mixtures of the antimicrobial peptide LL-37 and dipalmitoyl-PC, which fused into positionally weakly correlated bilayer sheets in the fluid phase (Sevcsik et al. 2007, 2008) (Fig. 8). There has been some debate about the peptide arrangement that surrounds the hydrophobic core of the bicelle, which will depend on the flexibility and particular length of the peptide. An orientation of the helix axis normal to the bilayer plane of the disk-like micelle was proposed for δ-lysin (Lohner et al. 1999) whereas location parallel to the disk-plane was suggested for LL-37

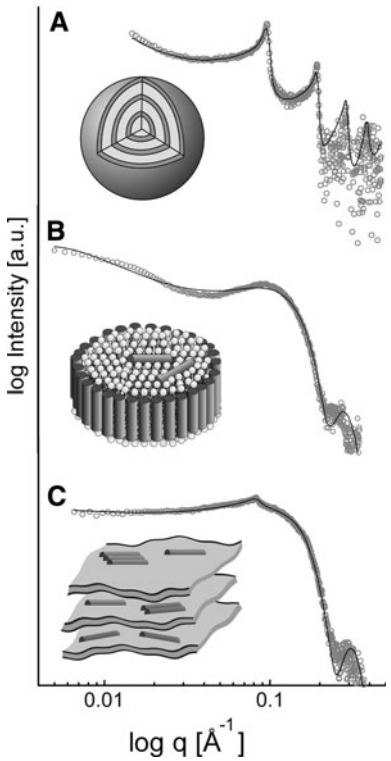

Fig. 8 SAXS pattern of pure dipalmitoyl-PC (DPPC) in the gel phase (**a**), DPPC with 4 mol% LL-37 in the gel (**b**) and fluid (**c**) phases (Sevcsik et al. 2007). Below the scattering pattern, sketches of the corresponding macroscopic phases are shown: Multilamellar vesicles (**a**), disk-like micelles (**b**), and extended bilayer sheets (**c**)

(Sevcsik et al. 2007), as shown for discoidal micelles formed by apolipoproteins (Atkinson and Small 1986). Recently, anomalous reflectivity using synchrotron radiation in combination with iodine-labeled peptides was used to reveal the position of the labeled amino acids (Schneggenburger et al. 2011) within the lipid bilayer. In the case of multiple labeling this approach will furnish information on peptide orientation relative to the lipid bilayer.

Drug-delivery systems

Plain liposomes for drug-delivery are mostly produced from synthetic phospholipid molecules and cholesterol to form lamellar lipid bilayer structures. In the aqueous interior of these vesicular carriers water-soluble pharmaceuticals can be entrapped, whereas amphiphilic or lipophilic drugs are, instead, accommodated in the lipid membrane or are tightly associated with the lipid surface. In this way, liposomes can serve as transport vehicles for proteins, small organic drugs, vaccines, oligonucleotides, or DNA. Appropriate properties of liposomes can be achieved by altering the lipid composition, e.g. by adding anionic or cationic phospholipids, co-additives, or pharmaceutical excipients to the formulation either before or after preparation. Because most pharmaceutical liposomes are intended for parenteral administration, they must be of a specific size: not too big to be readily cleared by the reticuloendothelial system, and not too small to incorporate an efficient amount of drugs. Given these facts, typical pharmaceutical liposomes have diameters in the range between 80 and 150 nm. They might be formulated as multilamellar vesicles, although most of the formulations are unilamellar vesicles with a single bilayer structure. An important disadvantage of liposomal drug-delivery systems is poor plasma stability. One way of overcoming this problem is to coat the liposome with a hydrophilic polymer, for example poly(ethylene glycol) (PEG), to create an impermeable protective shield, because of steric repulsion by the high flexibility of the surface-grafted polymers. Such sterically stabilized or "stealth liposomes" have prolonged circulation times in blood and are widely used for biomedical applications (Crommelin et al. 2003).

X-ray scattering experiments on pharmaceutical liposomes

Pharmaceutical liposomes are approximately 100 nm in diameter and consequently too large to be "seen" directly by SAXS. Biophysical characterization of pharmaceutical liposomes is therefore focused on dynamic light scattering and electron microscopy to determine size, and morphology or crystallinity of incorporated drugs. SAXS is needed to study the internal organization and bilayer structure. Because pharmaceutical formulations mostly comprise large unilamellar vesicles, a diffuse X-ray scattering pattern is obtained, which enables determination of characteristic bilayer parameters, e.g. bilayer thickness. This parameter is clearly affected by the lipid composition and reflects any changes that occur upon bilayer modification. For example, SAXS can be used to study the effect of cholesterol on membrane properties (Hodzic et al. 2008), which is an important excipient in pharmaceutical formulations. The cholesterol content might be extremely high. Some formulations contain as much as 40 mol% cholesterol with the purpose of making the bilayer more dense and rigid, and hence less permeable to encapsulated drugs. Some of these drugs, especially protein drugs, may in turn interact with the bilayer, affecting its integrity or physiological performance.

As mentioned above, stabilization of drug-delivery systems by surface coating with poly(ethylene glycol) is an interesting issue. This can be achieved most easily by using PEGylated lipids e.g. poly(ethylene glycol), covalently attached to phosphatidylethanolamine. Studies have already shown that the concentration of PEG–lipids and the mean molecular weight of PEG chain are critical

parameters in the synthesis of an appropriate delivery system (Woodle and Lasic 1992). If the PEG–lipid concentration is too low, the shielding effect is lost, because the polymer chains are separated from each other ("mushroom" conformation). With increasing PEG concentration the surface coverage is high enough that the neighboring PEG coils interact laterally. Then polymer chains are in the so-called "brush" conformation. Further increase in polymer concentration induces a series of morphological transformations, for example to disk-shaped aggregates, rod-like micelles, and spherical micelles. To study these transformations in more detail, combined SAXS and small-angle neutron scattering (SANS) studies have been conducted, exploiting density contrast variation (Arleth et al. 2005). The results, discussed in terms of the well-accepted critical packing parameter theory for surfactant systems (Israelachvili and Mitchell 1975), suggested that the transformation from vesicles to micelles is driven by changes in the spontaneous curvature of the system, because PEGylated lipids have a larger intrinsic curvature. The liposome-to-micelle transition was also apparent from dynamic light scattering and cryo-transmission electron microscopy (Edwards et al. 1997; Johnsson and Edwards 2003).

Biophysical studies on PEGylated multilamellar vesicles, combining osmotic pressure and X-ray diffraction techniques, have given a clearer picture of the conditions necessary to preserve lamellar bilayer structures for surfaces completely covered by overlapping polymer chains, that provide a steric barrier at the same time (Kenworthy et al. 1995a, b). Focusing on the physicochemical properties of the polymer chains, PEG-grafted liposomes were described by mean-field and scaling theories (comprehensively reviewed by Marsh et al. 2003). Briefly, PEG–lipids with a polymer molecular weight of 1 or 2 kDa are most effective in stabilizing liposomes at PEG–lipid concentrations of approximately 3–5 mol%. Issues concerning the distribution and thickness of the polymer coat were addressed recently by scattering techniques (Arleth and Vermehren 2010). Their results support an analytical model for PEG-grafted liposomes in which the liposomes are described as a water core surrounded by a lipid bilayer with polymer chains in a Gaussian random coil conformation. In this study an even distribution of PEG chains in the inside and the outside of the liposomes is assumed. The data are expressed analytically in terms of liposome radius, polydispersity, bilayer thickness, and radius of gyration of the PEG chain. Most recently, Varga et al. (2012) used synchrotron SAXS to analyze the PEG-shell coating of unilamellar vesicles describing the electron density profile of the bilayer with a superposition of Gaussian functions. The results from the electron density profiles are found to be in good agreement with theoretical predictions. For the liposomal formulation, which most closely resembles the pharmaceutical product doxil, the authors reported an asymmetric distribution of the PEG-2000 lipids, with thicknesses of the polymer layers of approximately 4.8 and 5.1 nm attached to the inside and the outside of the lipid leaflet, respectively. Approximately 75 % of the PEG–lipids were assigned to the outer membrane layer. For PEG with a molecular weight of 1 kDa (PEG-1000) the thickness of the outer layer is reduced to 2.7 nm, with a symmetric distribution of the PEG–lipids.

Another important aspect of liposomal drug-delivery systems is their long-term stability and the establishment of proper storage conditions, which can be achieved by lyophilization of the liposomes in the presence of sugars as cryoprotectants. In this respect it is highly important to ensure the structural integrity of the reconstituted liposomal formulation after rehydration. Along with other techniques, these features can be monitored by SAXS measurements (Stark et al. 2010), looking at the bilayer organization (Fig. 9).

Outlook

With the development of global X-ray data-analysis techniques it became feasible to analyze the scattering patterns of fully hydrated liposomal dispersions in the fluid phase, irrespective of the internal structure of the vesicles (multilamellar, oligolamellar, or unilamellar) (Pabst et al. 2010). Because of the complexity of natural membranes, phase separation is an important problem that must also be tackled by use of global analysis procedures. Phase separation may occur or be changed, because of interactions between lipids, proteins, and drugs. In particular antimicrobial peptides are known to induce phase separation into peptide-enriched and peptide-poor domains (Lohner and Blondelle 2005). Hitherto, phase-separated domains were detected by X-ray scattering in the gel phase, facilitated by the large difference in membrane thickness between lamellar gel and quasi-interdigitated phases. It will be more challenging to detect these domains in the fluid phase also, in which differences in membrane thickness are less significant, but our efforts are directed in this direction. Questions, we will be able to answer in the future by use of X-ray scattering are not how compounds change membrane structure or elasticity, but how they affect the structure and elastic properties of lipid domains of a given lipid architecture and how this couples to membrane functionality.

Such developments will necessitate a combination of X-ray work with neutron scattering experiments and molecular dynamics simulations, but also spectroscopic techniques, providing complementary information on domain stability and on drug location and orientation in

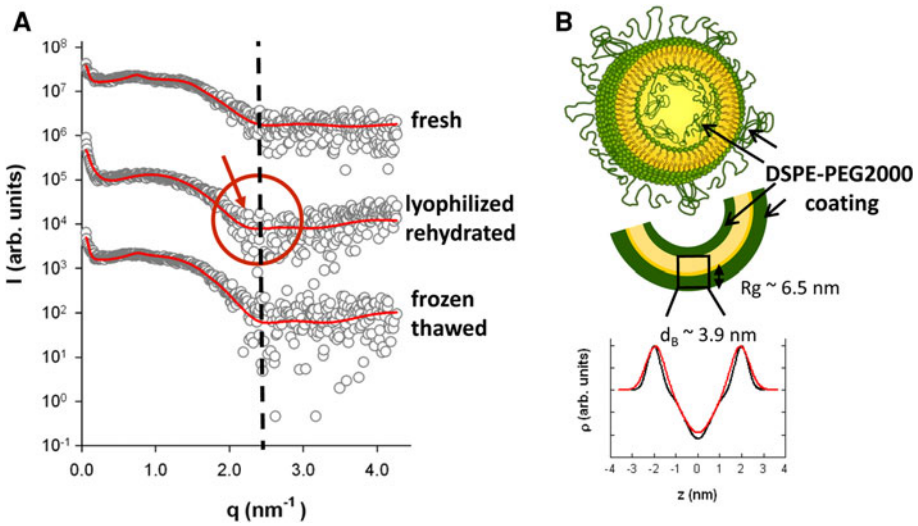

Fig. 9 Storage conditions for a PEGylated liposomal formulation were established by freezing followed by lyophilization in the presence of trehalose (Stark et al. 2010). **a** While the structural integrity of the thawed sample was preserved, the SAXS pattern of the rehydrated sample revealed swelling of the bilayer, indicating that the concentration of trehalose was not sufficient to protect the liposomal formulation during drying steps. **b** From the electron density profile the bilayer thickness (d_B) can be calculated by a global fit. A radius of gyration (R_g) is determined from the scattering curve in the absence of sugars and can be attributed to the contribution of the PEG chains

membranes. This will enable us to progressively increase the complexity of the lipid mixtures thus closing the gap to cell biology. The objective is to develop lipid-based model systems that, on the one hand, can be controlled stringently by experiments, but, on the other hand, have essential features of natural membranes, for example domain formation and protein partitioning. Finally, knowledge obtained about domain structure and elasticity of complex lipid mixtures will also assist future developments of more complex multifunctional delivery systems. Thus, X-ray experiments will remain invaluable in fundamental biological membrane research with applications to drug design and delivery.

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