

## Resolving the kinetics of lipid, protein and peptide diffusion in membranes

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### Abstract

Recent developments in the understanding of molecular diffusion phenomena in membranes are reviewed. Both model bilayers and biological membranes are considered in respect of lateral diffusion, rotational diffusion and transverse diffusion (flip-flop). For model systems, particular attention is paid to recent data obtained using surface-specific techniques such as sum frequency generation vibrational spectroscopy on supported lipid bilayers, and fluorescence correlation spectroscopy on giant unilamellar vesicles, both of which have yielded new insights into the intrinsic rates of diffusion and the energetic barriers to processes such as lipid flip-flop. Advances in single-molecule and many-molecule fluorescence methodologies have enabled the observation of processes such as anomalous diffusion for some membrane species in biological membranes. These are discussed in terms of new models for the role of membrane interactions with the cytoskeleton, the effects of molecular crowding in membranes, and the formation of lipid rafts. The diffusion of peptides, proteins and lipids is considered, particularly in relation to the means by which antimicrobial peptide activity may be rationalized in terms of membrane poration and lipid flip-flop.

**Keywords:** *Kinetics, peptide-lipid interactions, lipid biophysics, membrane model, flip-flop*

### Introduction

Lipid membranes are a fundamental component of biological systems, constituting both the barriers that maintain cell integrity and the partitions by which cells are divided into compartments with distinct biological and physiological properties. Our view of the biological membrane has changed significantly since the fluid mosaic model was developed by Singer and Nicolson (Singer and Nicolson 1972). Whilst the salient features of their model hold true, such as the innate ability of phospholipids to form bilayers and the potential for membrane proteins to diffuse within the bilayer, many of the now commonly recognized features of biological membranes are absent, including the inhomogeneous distribution of lipids and the presence of essentially static proteins that are associated with the cytoskeleton. The majority of biological membranes are a chemically diverse cocktail of lipids and proteins, with a broad range of lipid headgroups and acyl chains, alongside proteins that may constitute greater than 50% of the mass of the membrane. Membranes are

frequently asymmetric, with non-uniform distributions of proteins and lipids both between leaflets and within a single leaflet. This asymmetry reflects the intrinsically dynamic nature of the membrane. Components are continually moving within the bilayer, forming complexes that may be long-lived, such as protein-protein adducts, or relatively short lived, such as protein-lipid or lipid-lipid adducts. Molecules are continually being recruited to or lost from the membrane, either as part of normal physiological processes such as signalling, or during recycling of membrane components. Understanding the rates by which peptides and proteins move within the membrane is therefore of fundamental importance for understanding a number of processes, including cell signalling, membrane poration, membrane fusion and the formation of lateral heterogeneity such as lipid rafts. Three general molecular processes can be described for membrane proteins and lipids: lateral diffusion in the plane of the membrane, rotation, and translocation between membrane leaflets. Other processes, such as molecular rocking or wobbling motions (Pastor et al. 2002, Pu et al. 2009) may

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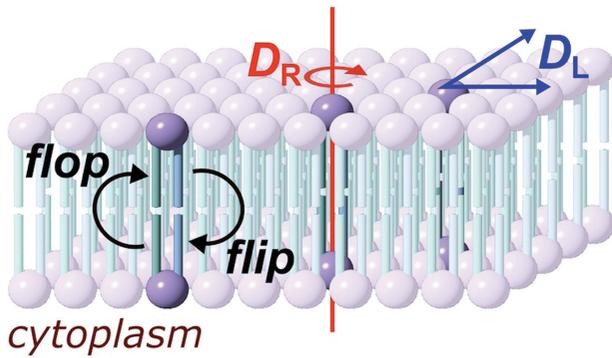


Figure 1. Diffusion processes of membrane molecules: transverse diffusion (interleaflet exchange; flip-flop), lateral diffusion (characterized by the coefficient  $D_L$ ) and rotational diffusion (characterized by the coefficient  $D_R$ ). This Figure is reproduced in color in *Molecular Membrane Biology* online.

also be considered, but have been less widely studied. Lateral diffusion, rotation and translocation are characterized respectively by the diffusion coefficient ( $D_L$ ), the rotational coefficient ( $D_R$ ) and the half-life for translocation ( $t_{1/2}$ ) (Figure 1). For each of these processes, this review summarizes recent developments in the methodologies available for the study of their kinetics and the insights that are emerging from the use of these techniques. Model systems are widely used to understand the fundamental aspects of membrane kinetics. The key advantage of model systems is that most of the complexity inherent to biological membranes is simplified or removed, with the consequence that important principles governing membrane activity can be revealed systematically. This review will cover both the fundamental aspects of membrane kinetics revealed using model systems and how these aid our understanding of kinetic processes in biological systems.

### Lateral diffusion

Constraining factors for performing meaningful measurements on lateral diffusion rates are the ability to obtain data with sufficient temporal resolution to capture all diffusion phenomena, and the effects of labels introduced to facilitate spectroscopic measurements, which may perturb membrane fluidity, in terms of both bulk membrane viscosity (fluidity being inversely proportional to viscosity) and the lateral diffusion of individual components. A number of enhanced nanoscopy methods have been used to study the distribution of components in the lipid membrane and have been well reviewed (Duggan et al. 2008). These are not generally applicable to quantifying lipid dynamics, and are therefore not

covered in detail here. Some techniques, most notably high-speed AFM, have not yet been widely adopted for quantifying diffusion within the membrane, but are likely to become increasingly important (Fantner et al. 2010, Casuso et al. 2011). Methods for studying lateral diffusion in the membrane have been reviewed (Kusumi et al. 2010, Owen et al. 2010) and the salient features of the principal methods used in the literature will be discussed first.

### Model systems and methods for studying lateral diffusion

#### *Supported lipid bilayers (SLBs)*

SLBs consist of a lipid bilayer adsorbed on the surface of a suitable solid substrate, such as gold, mica or silicon dioxide (Castellana and Cremer 2006, Czolkos et al. 2011), and are finding increasing usage both for studying lateral diffusion and interleaflet exchange of lipids. The choice of substrate and deposition method is determined by the requirements of the experiment in question. Three methods are generally used for preparing SLBs: Langmuir-Blodgett (LB, Figure 2A) or Langmuir-Schaeffer (LS, Figure 2C) deposition, vesicle fusion (VF, Figure 2B), and a hybrid of the two (LS/VF, Figure 2D), where vesicles are fused onto an existing monolayer. LB deposition involves the transfer of successive lipid monolayers from a suitable interface (e.g., air/water) to the solid surface by drawing the surface through the interface, and is well-suited to the preparation of model asymmetric membranes. The key advantages of this method are that the composition of each layer is easily controlled and deposition can be conducted at a controlled surface pressure, giving access to the fundamental thermodynamic parameters associated with lipid translocation. VF involves treating the surface with a liposome preparation and allowing the vesicles to fuse on the surface to form a complete layer. Whilst this method is the most convenient, it does not readily facilitate the formation of asymmetric bilayers unless combined with LS deposition of a single monolayer. It can be argued that the nature of the SLB, where one leaflet is closely associated with the solid support, is not a good representation of a biological membrane. However, neutron reflectometry experiments reveal that a 10–20 Å water layer containing ions remains trapped between the surface of the lipid layer and the solid support, and lateral mobility in the proximal leaflet is preserved (Johnson et al. 1991), indicating that the bilayer retains many of the properties of a free membrane. The spontaneous generation of lateral asymmetry in a bilayer formed by vesicle fusion

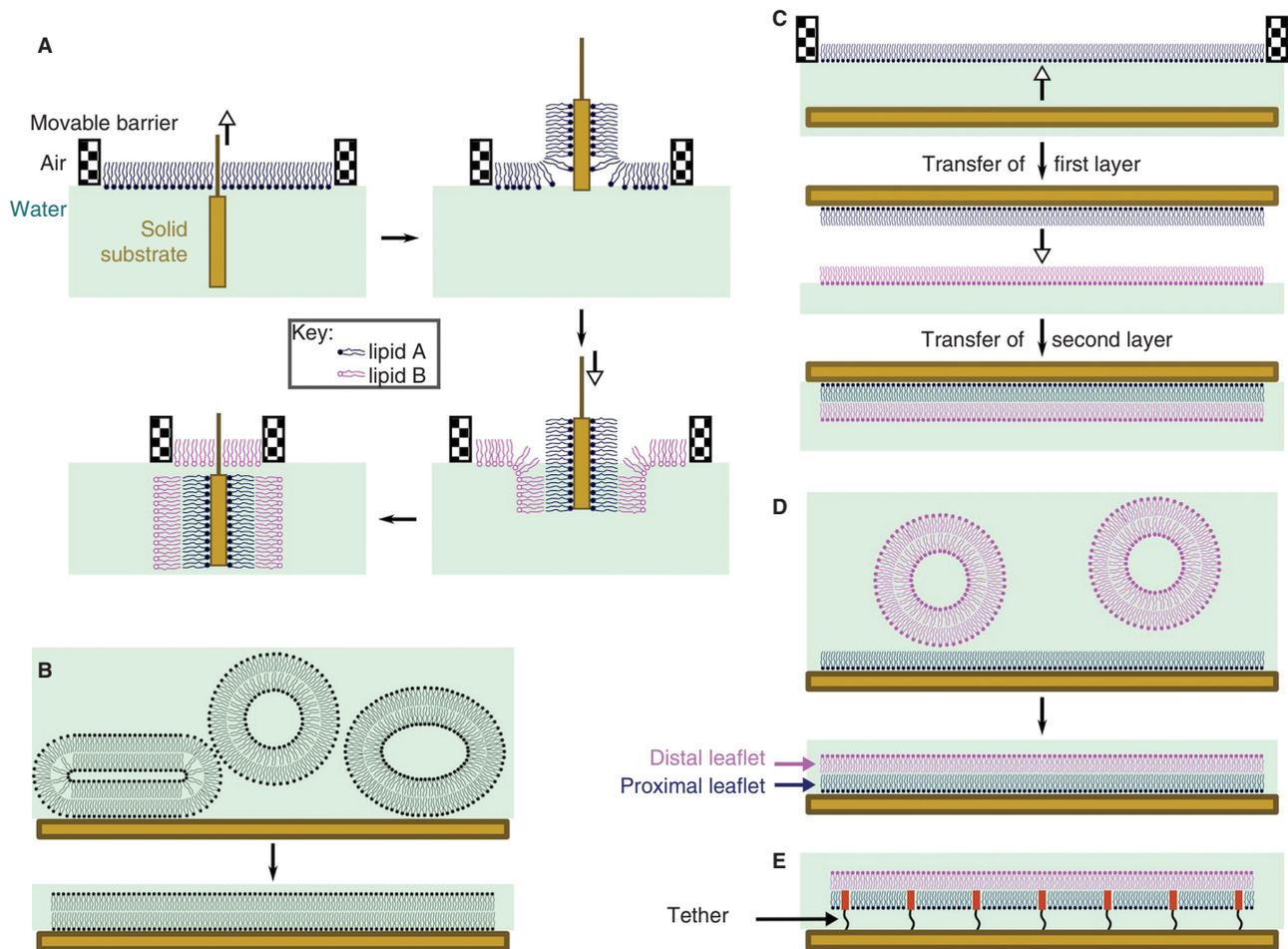


Figure 2. Methods for the preparation of supported lipid bilayers (SLBs). (A) In the Langmuir-Blodgett (LB) method, the solid substrate is drawn through a monolayer of one lipid (lipid A) and subsequently pushed through a second layer (lipid B), producing an asymmetric layer. Each monolayer is at a controlled area per lipid molecule and surface pressure, giving excellent control of the composition of the SLB; (B) Vesicle fusion (VF) is the simplest method, but is not useful for the preparation of asymmetric bilayers; (C) In the Langmuir-Schaeffer (LS) method, entire intact monolayers are transferred to the solid substrate in successive operations; (D) Hybrid LS/VF or LB/VF methods. These allow asymmetric bilayers to be prepared *in situ* and are ideal for conducting measurements on SLBs immediately after preparation; (E) Tethered or polymer-supported bilayers consist of an amphiphile anchored to the surface of the substrate by a polymer (e.g., polyethyleneglycol), around which the proximal monolayer is formed. Tethered bilayers have a greater water layer depth between the proximal surface of the bilayer and the solid support. This Figure is reproduced in color in *Molecular Membrane Biology* online.

has recently been reported (Wacklin 2011), presumably due to the specific interactions of some lipids with the surface, with the consequence that the chemical identity of each of the membrane leaflets cannot be assumed with SLBs prepared in this manner. In part because of concerns about the interaction of the proximal leaflet with the substrate surface, the use of a polymer cushion between the solid support and the bilayer has become more common (Figure 2E), providing for a greater water layer depth (Castellana and Cremer 2006, Czolkos et al. 2011).

#### Fluorescence recovery after photobleaching (FRAP)

FRAP requires the membrane to be labeled with a suitable fluorophore. An incident beam of laser light is

focused on the membrane, leading to photobleaching of the fluorophore within the focal spot. The rate of diffusion of non-bleached fluorophores into the bleached area is then monitored. The main drawbacks with this method are that photobleaching is not instantaneous and may not always be fully irreversible, with the consequence that some molecules will diffuse into the spot during the bleaching process (Weiss 2004). These factors, together with the flickering behavior typical of some fluorophores, introduce error into the measurements (Periasamy et al. 1996). Improved accuracy can be achieved by using spots of varying size to account for marker ingress during photobleaching, using pulsed lasers (van den Bogaart et al. 2007), or through the use of laser beams with modified profiles (Berkovich et al. 2011).

Two-color methods, demonstrated using the photo-convertible protein dendra2, offer the possibility of simultaneously monitoring marker ingress during fluorescence recovery (of the green form of the dendra2) and marker egress from the irradiated area (of the red form of the dendra2), providing useful controls for modelling the data (Kaya et al. 2011).

#### *Single fluorescent molecule tracking (SFMT)*

Historically, monitoring the movement of a single molecule bearing a fluorescent marker was challenging, principally because the period in which the fluorophore could be tracked before photobleaching occurred was short and detectors lacked the sensitivity required. More recently however, the advent of new chromophores, allied to improvements in detector sensitivity, has made SFMT a feasible process (Kusumi et al. 2010, 2011, Rolfe et al. 2011). A key requirement for SFMT is to track the movement of single molecules with a high enough frame rate to capture sufficient data for analysis (Skaug et al. 2011). Frame rates of 10–1000 per second are typical, with a spatial resolution of 30–40 nm.

#### *Single particle tracking (SPT)*

SPT is typically conducted using either colloidal gold particles of diameter 20–100 nm (Eisenthal 2006), or quantum dots (QDs) of 2–10 nm diameter, composed of CdSe or ZnS (Biju et al. 2010, Pinaud et al. 2010). Methods involving gold particles rely on the scattering of incident light to generate interference contrast, producing high signal-to-noise ratios and excellent spatial resolution (2–20 nm, depending on frame rate). QDs are fluorescent, with the emission wavelength tuneable according to their diameter providing opportunities for multi-color labeling (Roullier et al. 2009). Importantly, QDs are very photostable. The movement of a single lipid or protein labeled with a QD can be tracked over long time periods (seconds to minutes) and large length scales (tens of micrometers). QDs are large in relation to the molecules to which they are attached. It has been estimated that attachment of a quantum dot is equivalent to adding an extra protein domain of ~500 kDa to the molecule (Schneider et al. 1998). This will have some effect on the rate of lateral diffusion, although the 2-dimensional diffusion of membrane proteins is modelled well by Saffman-Delbrück theory (Saffman and Delbrück 1975, Mika and Poolman 2011), in which the diffusion coefficient varies according to the logarithm of the inverse of the radius of the section of the protein embedded within the membrane (i.e.,  $D_L \propto \ln(1/R)$ , where  $R$  is the radius of the embedded section of the

protein). As a consequence, protein dimerization for example, will produce a change in  $D_L$  of ~10%. Furthermore, as the viscosity of the membrane is significantly higher than the bulk medium (water), the attachment of a large water-exposed group to the membrane protein only has a small effect on  $D_L$ . In some cases, attachment of QDs to transmembrane proteins has been found to produce anomalous diffusion (Nechyporuk-Zloy et al. 2008). Furthermore, in some cases QDs have demonstrated a tendency to self-associate to form clusters (Kusumi et al. 2010). There is therefore some potential for modification with a QD to hinder the diffusion of the labeled molecule. As with SFMT, the frame rate has an important influence on the quality of the data obtained, as low frame rates cannot capture localized variations in diffusion.

#### *Fluorescence correlation spectroscopy (FCS)*

FCS measures the fluorescence intensity in the focal spot of a laser as a function of time. From the autocorrelation function (Noda 2010), the fluorophore density and residence time in the spot are determined. FCS, like FRAP, is a many molecule technique, requiring data to be averaged for a large number of molecules to achieve good signal-to-noise ratios (Kusumi et al. 2010, Melo et al. 2011). In FCS, the minimum size of the focal spot (~200 nm) limits the spatial resolution, which has led to the development of STED-FCS, in which the size of the focal spot is reduced below the optical diffraction limit to ~30 nm. This in turn leads to improved modelling of the data to extract lateral diffusion coefficients (Mueller et al. 2011).

#### *Pulsed field gradient nuclear magnetic resonance spectroscopy (PFG-NMR)*

This method is commonly used for probing dynamics in model lipid systems such as liposomes. Unlike the methods described above, this technique can be performed without the introduction of labels, and in ideal cases can distinguish individual components of the membrane, making it a valuable analytical method. The membranes to be studied need to be aligned with respect to the experimental frame of reference, which may be achieved using bilayers formed on glass plates, or by using magnetically aligned bicelles (Horst et al. 2011, Macdonald and Soong 2011). During the signal acquisition sequence, two pulsed magnetic fields are applied at different times that lead to changes of intensity for molecules that have moved between the pulses, with the magnitude of the change dependent on the extent to which molecules have

moved, i.e.,  $D_L$ . Limitations of this approach include the line broadening associated with the NMR spectra of lipid membranes, which may require the experiments to be conducted at the magic angle and limits the resolution that can be obtained, and the difficulty in distinguishing membrane components such as cholesterol that only have  $^{13}\text{C}$  and  $^1\text{H}$  nuclei available.

## Measurements of $D_L$ in model systems

### *Single component lipid systems*

For simple membranes formed from single lipid components in the fluid phase at room temperature, such as DOPC or POPC, values for  $D_L$  in the range  $5\text{--}8\ \mu\text{m}^2\ \text{s}^{-1}$  are typical when determined by FCS methods on giant unilamellar vesicles (GUVs), regardless of the fluorescent species that is monitored (Kahya et al. 2003, Kahya and Schwille 2006, Przybylo et al. 2006, Ariola et al. 2009). Similar values are obtained by PFG-NMR (Orädd et al. 2002, Filippov et al. 2003, Lindblom et al. 2006). However,  $D_L$  values obtained by FCS on supported lipid bilayers (SLBs) tend to be lower by a factor of 2–5 when compared with free-standing vesicle membranes of similar composition, even for SLBs separated from the surface by a polymer cushion (Sonnleitner et al. 1999, Zhang and Granick 2005). In most cases, the differences in  $D_L$  between the bilayer leaflets on SLBs are small and within experimental error (5–10%), regardless of whether the SLB is formed directly on the solid support or on a polymer cushion (Wagner and Tamm 2000, Naumann et al. 2002, Zhang and Granick 2005), although in some cases  $D_L$  values for the proximal and distal leaflet have been reported to differ by an order of magnitude (Hennig et al. 2009). The lower  $D_L$  values for SLBs formed directly on a solid surface may be taken as an indication of a frictional interaction between the proximal (inner) leaflet and the surface, with similar  $D_L$  values for both leaflets of SLBs suggestive of frictional coupling between the leaflets (Przybylo et al. 2006).

As expected, diffusion coefficients increase as the temperature is raised, with  $D_L$  values of  $20\text{--}30\ \mu\text{m}^2\ \text{s}^{-1}$  reported for DMPC, DPPC, DOPC and POPC using PFG-NMR at  $60^\circ\text{C}$  (Filippov et al. 2003, Lindblom et al. 2006). Significantly slower diffusion coefficients are found for sphingomyelin (SM) membranes at room temperature, with values  $< 0.5\ \mu\text{m}^2\ \text{s}^{-1}$ , consistent with these membranes existing in the gel state at this temperature (Kahya et al. 2003, Ariola et al. 2009). Diffusion coefficients are higher in SM membranes at increased temperatures, although still considerably slower than fluid PC membranes, with

values in the range  $2\text{--}4\ \mu\text{m}^2\ \text{s}^{-1}$  at  $40\text{--}42^\circ\text{C}$  and  $9\text{--}12\ \mu\text{m}^2\ \text{s}^{-1}$  at  $60^\circ\text{C}$  (Filippov et al. 2003, Lindblom et al. 2006). Studies of multilamellar liposomal membranes by neutron scattering (Busch et al. 2010) and simulation (Falck et al. 2008) have found evidence for flow-like behavior, in which lipid molecules move collectively. It remains to be seen if this is a general phenomenon that needs to be considered when accounting for differences in lateral diffusion rates between model and biological membranes, particularly as long-range flow is likely to be restricted by interactions of the cell membrane with the cytoskeleton.

### *Binary and ternary lipid mixtures*

Complex mixing behavior is found for model membranes consisting of a ternary mixture of a lipid with a high gel to liquid crystal phase transition temperature (high- $T_m$  lipid), a lipid with a low gel to liquid crystal phase transition temperature (low- $T_m$  lipid) and cholesterol. Under appropriate conditions of temperature and composition, these membranes separate into macroscopic fluid liquid-disordered ( $l_d$ ) and condensed liquid-ordered ( $l_o$ ) domains (Veatch and Keller 2005, Honerkamp-Smith et al. 2009). The  $l_o$  domain is enriched in cholesterol and the high- $T_m$  lipid, with the low- $T_m$  lipid localized predominantly in the  $l_d$  domain. Although this macroscopic phase separation does not occur *in vivo*, the lipids involved in the formation of  $l_o$  domains *in vitro* are frequently isolated from detergent-resistant extracts of biogenic membranes (Lagerholm et al. 2005, Lichtenberg et al. 2005, Brown 2006), and the same lipids are proposed to be components of lipid rafts. For the purposes of this review, lipid rafts are defined as localized regions of heterogeneity in biological membranes that form dynamically on a small scale (diameter  $\leq 40\ \text{nm}$ ) (Lingwood and Simons 2010). It is therefore of intrinsic interest to quantify diffusion coefficients in model systems that exhibit domain formation. Ternary mixtures of DOPC, SM and cholesterol have been studied extensively by PFG-NMR and FCS. For mixtures with a DOPC/SM/cholesterol composition that is close to 1:1:1, at temperatures below  $25^\circ\text{C}$ , two separate diffusion constants are obtained for the  $l_o$  and  $l_d$  phases, with  $D_L$  values of  $0.2\text{--}0.8\ \mu\text{m}^2\ \text{s}^{-1}$  and  $3\text{--}6\ \mu\text{m}^2\ \text{s}^{-1}$ , respectively (Kahya et al. 2003, Lindblom et al. 2006, Ulrich et al. 2008). These values are in broad agreement with the diffusion coefficients for single component fluid and gel phase membranes, as well as binary mixtures of SM/cholesterol (1:1), which are in an  $l_o$  phase at cholesterol concentrations  $> 35\ \text{mol}\%$  (Filippov et al. 2003, Kahya and Schwille 2006). As the temperature of the

DOPC/SM/cholesterol ternary system is increased, the  $l_d$  and  $l_o$  phases coalesce to form a single phase with a diffusion coefficient ( $14 \mu\text{m}^2 \text{s}^{-1}$  at  $60^\circ\text{C}$ ) that is intermediate between those of fluid and gel phase membranes at the same temperature (Lindblom et al. 2006). Comparable diffusion coefficients for  $l_d$  and  $l_o$  phases have been obtained following microphase separation of ternary DOPC/DPPE/cholesterol mixtures (Lindblom et al. 2006); in this case the  $l_o$  phase is enriched in DPPE and cholesterol. Binary mixtures of DPPE/cholesterol similarly exhibit diffusion properties typical of an  $l_o$  phase ( $0.8 \mu\text{m}^2 \text{s}^{-1}$  at  $24^\circ\text{C}$ ) (Lindblom et al. 2006). Through the use of deuterium-labeled DPPE in binary mixtures with cholesterol it has been possible to determine separate  $D_L$  values for each of the components over a range of temperatures and compositions (Scheidt et al. 2005). In these binary mixtures, cholesterol concentrations  $< 35 \text{ mol}\%$  yield complex mixtures of  $l_o$ ,  $l_d$  and solid-ordered ( $s_o$ ) phases, whereas cholesterol concentrations  $> 35 \text{ mol}\%$  produce pure  $l_o$  phases. For most of these phases, including  $l_o$ , the diffusion coefficients of each component are found to follow similar trends, with cholesterol always diffusing slightly faster than DPPE. Similar effects have been observed using  $^{19}\text{F}$ -labeled cholesterol and have been attributed in part to an interaction between the lipid and cholesterol, and in part to the lower molecular weight of cholesterol with respect to the lipid (Orädd et al. 2002). As a general rule, diffusion coefficients decrease with increasing cholesterol content in membranes containing cholesterol and a single low- $T_m$  lipid. By contrast, diffusion coefficients are increased in binary mixtures with a high- $T_m$  lipid as the cholesterol content is raised (Filippov et al. 2003, Kahya and Schwille 2006, Day and Kenworthy 2009).

### Peptides and proteins

Studies that have employed labeled peptides or proteins on SLBs or GUV membranes have revealed interesting details of the effects of bilayer structure on lateral diffusion. A study on the effects of membrane curvature on the lateral diffusion of lipids and the potassium channel KvAP was conducted using SPT with quantum dots (Domanov et al. 2011). This yielded slower diffusion rates for the protein ( $D_L = 2.3 \mu\text{m}^2 \text{s}^{-1}$ ) when compared with the lipid ( $D_L = 3.3 \mu\text{m}^2 \text{s}^{-1}$ ), in accordance with Saffman-Delbrück theory (Saffman and Delbrück 1975). This study also demonstrated that  $D_L$  is inherently sensitive to the curvature of the membrane, with diffusion coefficients increasing in proportion to the logarithm of the diameter of a membrane tube pulled from the surface of a GUV. Experiments to probe the

effects of hydrophobic mismatch on the lateral diffusion of a transmembrane peptide have revealed that peptide mobility in GUVs formed from SOPC is greatest ( $D_L \sim 0.4 \mu\text{m}^2 \text{s}^{-1}$ ) when the length of the hydrophobic transmembrane segment matches the hydrophobic thickness of the bilayer (Gambin et al. 2010). Lipid diffusion coefficients in these experiments were in line with those described above for fluid phase lipids ( $D_L \sim 5 \mu\text{m}^2 \text{s}^{-1}$ ). Faster peptide diffusion was also obtained when the bilayer was of sufficient thickness that contact was reduced between a (non-transmembrane) peptide embedded in one monolayer and the lipids of the other monolayer (Gambin et al. 2010).

Incorporation of an amphipathic peptide fragment of hepatitis C virus non-structural protein 5A into an SLB formed from POPC was found to reduce the lipid diffusion coefficient from  $2.0 \mu\text{m}^2 \text{s}^{-1}$  to almost zero when studied by FRAP. Control experiments with a similar peptide of reduced amphipathicity yielded normal diffusion rates. On the basis of atomic force microscopy this reduced lateral mobility was attributed to membrane thinning induced by the peptide (Cho et al. 2007).

### Measurements of $D_L$ in biogenic membranes

Diffusion coefficients measured *in vivo*, for both lipids and proteins, are typically an order of magnitude smaller than those determined using model systems. For lipids,  $D_L$  is typically in the range  $0.1\text{--}0.9 \mu\text{m}^2 \text{s}^{-1}$  (Crane and Verkman 2008, Golebiewska et al. 2008, 2011, Baier et al. 2010, Mueller et al. 2011), with the corresponding values for peptides and proteins covering a greater range of  $0.001\text{--}0.2 \mu\text{m}^2 \text{s}^{-1}$  in most cases (Crane and Verkman 2008, Roullier et al. 2009, Baier et al. 2010, Won et al. 2010, Kaya et al. 2011, Valentine and Haggie 2011). These reduced diffusion rates have been attributed to two fundamental causes (Dix and Verkman 2008, Mika and Poolman 2011): (i) Molecular crowding; and (ii) membrane-cytoskeletal interactions. Molecular crowding is a reflection of the high protein content of most biogenic membranes, with integral membrane proteins occupying  $> 20\%$  of the area and  $\sim 20\%$  of the mass of the plasma membrane (Dupuy and Engelman 2008), extending to  $\geq 50\%$  of the mass in the inner mitochondrial membrane (Zinser et al. 1991). Molecular motion in these crowded membranes may therefore be restricted by the high protein content. Consistent with this theory, it has been demonstrated that  $D_L$  decreases linearly with respect to increasing protein concentration in GUV membranes (Ramadurai et al. 2009) and SLBs (Horton et al. 2010). Membrane-embedded proteins that interact with the cytoskeleton

naturally display restricted lateral diffusion (Haggie et al. 2006, Crane et al. 2008, Valentine and Haggie 2011). Membrane proteins anchored to the cytoskeleton have been implicated in the generation of anomalous diffusion patterns for other membrane molecules.

### *Anomalous diffusion*

Recent experiments in biogenic membranes have yielded complex patterns of lateral diffusion. In *normal* (Brownian) diffusion, the mean square displacement (MSD) of molecules increases linearly with regard to the length of the observation period: if the period of observation is doubled, the MSD also doubles. However, in some cases, the MSD of membrane components has been found to increase (superdiffusion) or decrease (subdiffusion) if the length of the observation window is increased (Dix and Verkman 2008), both of which may be described as examples of anomalous diffusion. In SPT experiments, anomalous diffusion is manifested by localized variations in the mean square displacement (Calvo-Muñoz et al. 2011). When monitored at low frequency, molecules appear to diffuse normally. In contrast, when monitored for sufficient periods at high frequency, the trajectories are divided into small localized regions (diameter ~30–300 nm) within which diffusion is normal ( $D_L = 0.1\text{--}0.6 \mu\text{m}^2 \text{s}^{-1}$  for lipids and transmembrane proteins at 37°C), with relatively infrequent ‘hops’ between adjacent regions (Kusumi et al. 2010, 2011, Crane et al. 2010, Valentine and Haggie 2011). The hop diffusion rate is the measured  $D_L$  (typically  $\leq 0.01 \mu\text{m}^2 \text{s}^{-1}$ ) when the sampling frequency of the trajectory is low. Slow hop diffusion between regions (compartments), within which diffusion is relatively fast, has been accounted for by picket-fence models, which include transmembrane proteins anchored to the cytoskeleton as the ‘pickets’ and membrane cytoskeletal proteins such as actin filaments as the ‘fence’ (Golebiewska et al. 2011, Kusumi et al. 2011). Lipid rafts may also account for some instances of anomalous diffusion and it is notable that the compartment size in the picket-fence models are of the same order of magnitude as lipid rafts (Kusumi et al. 2010, 2011). Anomalous diffusion is not a ubiquitous phenomenon. For example, the diffusion properties of some proteins, such as aquaporin-1 (Crane and Verkman 2008) and the nicotinic acetylcholine receptor (Baier et al. 2010), are normal and unchanged following actin depolymerization by latrunculin (Frick et al. 2007). Aquaporin-4 on the other hand, shows more complex behavior in COS-7 cells, with the M1 isoform showing normal diffusion and the M23 isoform, which is

able to assemble into slowly diffusing orthogonal arrays of particles, showing anomalous diffusion (Crane et al. 2010). Normal diffusion has also been reported for a number of G-protein coupled receptors (Kaya et al. 2011). As one would expect, the lateral diffusion behavior of a membrane protein is specific both to the cell in question and the region on the cell surface where the measurement is obtained. For example, QD-labeled BK<sub>Ca</sub> channels show anomalous diffusion in COS-7 cells and the somatal and axiodendritic regions of neuronal cells, with the diffusion coefficients differing by an order of magnitude (Won et al. 2010). In the ER, inositol 1,4,5-trisphosphate receptors have been shown to display differences in mobility and distribution according to subtype (Pantazaka and Taylor 2011). Lipidated proteins that are associated with detergent-resistant membrane extracts have been shown to change from anomalous diffusion to normal diffusion upon cholesterol depletion of the membrane, suggesting a role for cholesterol in modifying diffusive behavior (Delint-Ramirez et al. 2011).

### *Lipid rafts*

As described above, under appropriate conditions in model systems, macroscopic  $l_o$  domains are formed that are enriched in cholesterol and a high- $T_m$  lipid. In biogenic membranes however, macroscopic domains are not observed and the distributions of components that are commonly associated with rafts, such as GPI-anchored proteins and SM, appear to be homogeneous when studied by microscopy (Jacobson et al. 2007). Rather, domains enriched in SM and cholesterol form dynamically on a scale (<40 nm) that is smaller than the optical diffraction limit (Hancock 2006, Jacobson et al. 2007, Lingwood and Simons 2010). These smaller domains frequently contain transmembrane, GPI-anchored or lipidated proteins, and are ‘primed’ to coalesce into larger domains during cell signalling (Lingwood and Simons 2010) or under the influence of membrane tension (Ayuyan and Cohen 2008). It is notable that this scale is also smaller than the size of the compartments in the picket-fence model. Much of the evidence for raft formation arises from the partitioning and diffusion behavior of probes. The choice of probe is not always trivial; for example, many labeled SM and cholesterol analogs partition into  $l_d$  rather than  $l_o$  domains due to the steric bulk of the modification (Wang and Silvius 2000, Shaw et al. 2006, Baumgart et al. 2007, Loura et al. 2009). In some cases the properties revealed by a probe, such as slow diffusion, may arise from the effects of anomalous diffusion combined with a sample rate that is too slow (for SPT) or

inappropriate modelling of data (for FCS) (Kusumi et al. 2010). The recent application of STED-FCS to the study of lipid dynamics within plasma membranes has led to improved spatial resolution and better modelling of the diffusion process (Mueller et al. 2011). This work accounted for the potential of probe modifications to modify partitioning behavior through the deployment of lipids labeled in either the headgroup of the acyl chain and highlighted the distinction between long-chain saturated lipids that diffuse slowly (in relative terms) and short-chain or unsaturated lipids that diffuse more rapidly. SM presented anomalous diffusion in the presence of cholesterol and normal diffusion following cholesterol depletion, with the rate of SM diffusion increasing in response to the reduction in cholesterol levels. Normal diffusion of SM was also observed after treatment with latrunculin B to induce actin depolymerization. Lipids bearing hydroxyl groups (gangliosides, PI) displayed an increased tendency to self-associate, but this was independent of cholesterol.

Overall, a picture is emerging in which small raft domains form dynamically in biogenic membranes through differences in the rate of association and dissociation of specific lipid types. Whether lipid diffusion between compartments is related to the formation of rafts is the subject of some debate (Kusumi et al. 2010, 2011, Mueller et al. 2011). A major role of cholesterol appears to be the regulation of membrane fluidity and lipid dynamics (Owen et al. 2010), respectively, retarding or accelerating the lateral diffusion of unsaturated and saturated lipids, as seen for PFG-NMR studies with binary cholesterol-PC mixtures (Filippov et al. 2003, Kahya et al. 2003, Kahya and Schwille 2006, Day and Kenworthy 2009).

### Rotational diffusion

In contrast to lateral diffusion, the rotational diffusion coefficient varies according to the inverse of the square of the radius of the embedded section of the protein, (i.e.,  $D_R \propto 1/R^2$ ) (Saffman and Delbrück 1975). As a consequence,  $D_R$  is a sensitive parameter for characterizing protein aggregation and protein-lipid interactions (Fooksman et al. 2007).

#### *Methods for determining $D_R$*

The most commonly used methods in the literature are ESR (Marsh 2008, Ryba and Marsh 1992, Mainali et al. 2011) and polarized optical methods (Swaminathan et al. 1997, Peters and Cherry 1982, Fooksman et al. 2007, Yengo and Berger 2010). NMR is useful for molecules that rotate relatively

slowly, such as peptides and proteins (Salnikow et al. 2010). A fundamental concern with determination of  $D_R$  is whether any probes that have been introduced to a lipid or protein are reporting the behavior of the molecule as a whole, or localized torsional rotations. For this reason, in many cases  $D_R$  measurements are made with the label incorporated at different positions in the molecule, such as the headgroup and acyl chains for lipids. Rotations about both the axis parallel to the membrane normal and the axis perpendicular to the membrane normal may be considered (Ge and Freed 2011).

#### *Typical values for membrane proteins and lipids*

In model gel phase membranes, lipids have a relatively low rotational diffusion coefficient of the order of  $10^6$ – $10^7$  s<sup>-1</sup>. The value of  $D_R$  increases in more fluid membranes, with reported values ranging from  $10^7$  s<sup>-1</sup> for  $l_d$  phases (Ariola et al. 2009), to  $10^9$  s<sup>-1</sup> in PC/PG/cholesterol membranes (Ge and Freed 2011). Membrane proteins generally yield rotational diffusion coefficients that are significantly reduced in comparison to lipids, with  $10^4$  s<sup>-1</sup> being typical (Cherry and Godfrey 1981, Peters and Cherry 1982), although fast rotation ( $D_R = 10^6$  s<sup>-1</sup>) has been reported for some peptides (De Angelis et al. 2011). The rotational diffusion of peptides is reduced to almost zero in gel phase membranes (Cornell et al. 1988). From a fundamental perspective, measurements of  $D_R$  for membrane proteins have yielded data both in support (Peters and Cherry 1982) and against (Ariola et al. 2009) Saffman-Delbrück theory descriptions of diffusion in membranes.

### Interleaflet lipid translocation (flip-flop)

It is now well-established that the plasma membranes of most cells are asymmetric with regard to the lipid composition of the cytoplasmic (inner) and extracellular (outer) leaflets. For example, in many eukaryotic cells, SM is enriched in the outer leaflet, whereas PE, PI and PS are predominantly located in the inner leaflet. The transmembrane distribution of lipids has been well reviewed (Pomorski et al. 2001, Boon and Smith 2002, Sanyal and Menon 2009, Devaux and Herrmann 2012). Transmembrane lipid distributions in eukaryotes are regulated by three main classes of enzyme: Floppases facilitate the movement of lipids from the inner leaflet to the outer leaflet, flippases facilitate the reverse translocation from the outer to the inner leaflet, and scramblases promote translocation in both directions (Daleke 2003, Devaux and Herrmann 2012). The proteins involved in lipid transport have proved particularly challenging to

characterize and there is still some debate as to whether flip-flop is an active (ATP-dependent) or passive (ATP-independent) process, or a combination of both. Some of the translocases involved in maintenance of asymmetry are ATP-dependent, most notably aminophospholipid translocases in the ER and  $P_4$ -ATPases in the plasma membrane (both flippases) (Verhulst et al. 2012). However, ATP-independent lipid translocation activity has been reported for microsomal membrane preparations that have been incorporated into liposomes, indicating that in the ER, energy-independent pathways for lipid flip-flop operate (Menon and Herrmann 2012). Much of the historical literature on transmembrane asymmetry is predicated on the propensity for lipids in the outer (but not inner) leaflets of biological membranes to undergo chemical reactions, or exchange with lipid vesicles (Etemadi 1980). Lipid exchange has traditionally been performed using labeled membrane lipids and unlabeled liposomes (LUVs) under catalysis by phospholipid exchange proteins (Rothman and Dawidowicz 1975, Lenard and Rothman 1976, Rothman et al. 1976), with the amount of lipid transferred to the liposomes quantified using standard analytical methods. These approaches work for a range of lipid types, although careful controls are needed to account for the lipid selectivity of the exchange protein. Methods employing enzymes that have lipids as substrates, such as phospholipase  $A_2$ , phospholipase C and sphingomyelinase offer improved selectivity (Boon and Smith 2002), but again careful controls are needed due to the potential for perturbation of the membrane as the reactions near completion (Wacklin et al. 2007). All of these methods work on the assumption that only the lipids in the outer leaflet are accessible, i.e., that the rate of flip-flop is slow with regard to the experimental timescale, and that the conditions of the experiment do not perturb asymmetry. This renders the study of flip-flop rates in membranes of fundamental significance. There are additional biological reasons for understanding the rate of flip-flop. In some organelles, such as the endoplasmic reticulum (ER), fast flip-flop is a key requirement for ensuring the availability of lipids that serve as substrates for protein modification (Devaux and Zachowski 1994, Sanyal and Menon 2009). ATP-independent translocation of most lipid types in these organelles occurs readily. By contrast, translocation of lipids in the plasma membrane exhibits greater lipid selectivity, which is allied to specific roles for some lipids in cell physiology, such as PI in secondary messenger signalling (Chakraborty et al. 2011), and PS appearance in the outer leaflet is a marker for apoptosis (Uchida et al. 1998).

The rate of flip-flop is conveniently expressed as the half-life for interleaflet transfer,  $t_{1/2}$ . Values for  $t_{1/2}$  range from short (s to min) for biological membranes such as the ER, to long (min to h) for the membranes of liposomes. For many years, following the classic experiment by (Kornberg and McConnell 1971) using spin-labeled diacylphosphocholine analogs, which established  $t_{1/2}$  values in the range 1.5–6.5 h at 30°C in synthetic membranes, the rate of flip-flop was generally accepted to be slow in biological membranes. More recently however, a number of studies on both model and biological membranes, using an array of alternative labeling strategies and spectroscopic approaches, have yielded a body of data that challenge the concept that flip-flop is always an inherently slow process. Three fundamental issues arise when considering these studies: firstly, it is clear that careful consideration of the effects of introducing a label on the translocation rate is needed; secondly, care is needed in assessing whether the method used is capable of delivering sufficient temporal resolution to resolve fast kinetic events; thirdly, it should be recognized that translocation rates are sensitive to the method used to prepare the membrane under investigation. As a consequence of these factors, comparisons of different experimentally determined flip-flop rates are challenging to make, even where the same lipid system has been studied. The methods most commonly used in the recent literature are outlined below.

### **Model systems and methods for the determination of flip-flop rates**

#### *Sum frequency generation vibrational spectroscopy (SFVS)*

Studies on SLBs have provided a rich body of data on the fundamental aspects of flip-flop, particularly when allied to SFVS. SFVS is a form of non-linear optical spectroscopy that provides similar information to Raman and infra-red spectroscopy (Liu and Conboy 2004, 2005, Eisenthal 2006, Yang et al. 2011). The method involves two lasers focused at the interface, one of which is operated at a fixed-wavelength in the UV range ( $\omega_{uv}$ ), the other being scanned over the IR frequency range ( $\omega_{ir}$ ). These two combine to form a signal at the sum frequency ( $\omega_{sum} = \omega_{uv} + \omega_{ir}$ ). When the sources are polarized and the sum signal focused at an appropriate surface, the incident beam is able to probe the orientation of surface molecules with sub-monolayer resolution. The output signals are in the IR frequency range and contain orientation information. When applied to bilayers adsorbed on the surface of a quartz crystal prism, signals from bilayer elements

that are symmetric about the plane of the membrane cancel each other out, rendering SFVS a particularly sensitive technique for studying membrane asymmetry. A further advantage of SFVS is that, in favourable cases, no labeling is required. Where labeling is needed, unobtrusive deuterium labels suffice to distinguish the membrane leaflets.

#### *Fluorescence-based methods*

These have the key advantage of being sufficiently sensitive and rapid to give excellent temporal resolution. However, the requirement for a component of the membrane to be modified with a non-biogenic fluorophore is a disadvantage. Translocation rates determined using fluorogenic lipids only reliably report the translocation rate of the labeled lipid in question, and not other classes of lipids of the membrane in which they are located. Methods used specifically to address bilayer asymmetry include fluorescence inhibition, by quenching or oxidation of the fluorophore in one leaflet by the addition of exogenous agents to one side of the membrane (Eckford and Sharom 2010, Pomorski et al. 2001), time-resolved emission spectroscopy (TRES) (Horng et al. 1995, Volinsky et al. 2011), fluorescence lifetime (Kułakowska et al. 2010) and fluorescence-interference contrast microscopy (FICS) (Crane et al. 2005).

#### *Specific binding*

In some cases, proteins that interact specifically with a particular class of membrane lipid can be used probe the appearance of that lipid in the outside leaflet of a cell or vesicle. This is most readily demonstrated by annexin V and lactadherin, which bind selectively to PS (Metkar et al. 2011) and pleckstrin homology domains, which have binding selectivity for PI lipids (Hurley and Meyer 2001, Hurley 2006).

#### **The debate over flip-flop: fast or slow?**

The breakthrough work by Kornberg and McConnell established lipid exchange in vesicles with a  $t_{1/2}$  in the range of 0.7–3 h (at 40°C), with an activation energy barrier to translocation ( $E_a$ ) in the range 65–116 kJ mol<sup>-1</sup> (Kornberg and McConnell 1971). Since then, a plethora of papers have published  $t_{1/2}$  data for most lipid types, with values spanning a huge range, from ms to days. It is not the intention to reproduce all of these data here, but salient examples will be selected to highlight the issues with interpretation of the data, and give a sense of typical values for common lipid types.

A series of SFVS experiments by Conboy have determined the rates of flip-flop in PC membranes on silica surfaces. Determination of the effects of temperature on flip-flop rates has revealed some of the fundamental thermodynamic aspects of the process in these systems. In general, longer chain PCs are found to undergo flip-flop more slowly than shorter chain counterparts, with a value for  $E_a$  of ~220 kJ mol<sup>-1</sup> for DPPC being typical. The free energy barrier to translocation ( $\Delta G^\ddagger$ ) is typically of the order of 100 kJ mol<sup>-1</sup> and includes a particularly large positive entropic component ( $T\Delta S^\ddagger \approx 130$  kJ mol<sup>-1</sup>) (Liu and Conboy 2005, Anglin et al. 2010). Most strikingly, the rates of flip-flop in these experiments are significantly faster than observed in biological membranes, with  $t_{1/2}$  in the range of seconds to minutes, e.g.,  $t_{1/2} = 52$  min for DPPC at 25°C and 30 mN/m surface pressure. The rate of flip-flop was found to be the same in both directions and the bilayers exhibited normal lateral diffusion rates, appeasing to some extent concerns that interaction of the bilayer with the solid support was responsible for the fast translocation rates. However, similar experiments by SFVS using asymmetric DPPC/dDPPC bilayers at 24°C yielded little exchange over course of an hour (Yang et al. 2011). Studies using FICS on polymer-tethered SLBs composed of POPC at 22°C (Kiessling et al. 2006) also gave significantly larger values for  $t_{1/2}$  (15 h), which is particularly notable as flip-flop is expected to be faster in the fluid phase POPC bilayer than the gel phase DPPC bilayer at this temperature. However, the lateral diffusion coefficient ( $D_L$ ) of labeled lipids was lower in this system than comparable examples with the same lipid composition. Of further note, a lipid with the same TEMPO spin label as the Kornberg and McConnell experiments was shown to undergo slower flip-flop in the Conboy SLB experiments than the same lipid when unmodified (Liu and Conboy 2005). This raises the question of whether the rate observed by Kornberg and McConnell is a true reflection of the rate of flip-flop in membranes, or represents an artifactually slower rate due to the presence of the lipid modification.

Another salient example of diverse  $t_{1/2}$  values has been provided by the examination of flip-flop rates in SLBs formed from DOPC/DOPS on ITO-treated glass (Kułakowska et al. 2010). Flip-flop in this system was addressed using fluorescent lipid analogs (Atto633-DOPE and F2N12S), with the variation in fluorescence lifetime (varying inversely with the distance from the ITO surface) used to assess flip-flop. Both of the labeled lipids gave similar and normal lateral diffusion rates (5–7  $\mu\text{m}^2 \text{s}^{-1}$ ), but flip-flop rates that differed by an order of magnitude according to the fluorescent lipid used ( $t_{1/2}$  values of

3 min for F2N12S and 32 min for Atto633-DOPE). Furthermore, F2N12S flip-flop in this system was significantly faster than found with the same label in biological membranes ( $t_{1/2} > 1$  h).

Studies directed to assaying the flip-flop of PE in the presence of other lipids have demonstrated that flip-flop occurs readily for this lipid, at similar rates to PC (Anglin and Conboy 2009). Flip-flop of PS is also well established (Langer and Langosch 2011, Volinsky et al. 2011), including a salient example of PS externalization within a period of  $< 5$  min on cytotoxic lymphocytes and mouse CD8 cells, following administration of the protein perforin at concentrations at which the protein associated with membranes as a monomer (Metkar et al. 2011). Equinatoxin II, a peptide toxin, was found to induce a similar PS externalization in the same cells. Of all the molecules found in eukaryotic membranes, cholesterol has produced the greatest variation in determined flip-flop rates. At the fast end of the spectrum, Müller and Herrmann used nitroxide-labeled sterols in order to probe flip-flop in synthetic vesicles and erythrocytes (Müller and Herrmann 2002). Reduction of the spin-label by ascorbic acid was used to assess label exposure on the external leaflet, in a manner reminiscent of the Kornberg and McConnell experiments. Significantly, for one of their analogs (a cholestane), exchange occurred at a faster rate than reduction, which only enabled an upper limit to be placed on the process ( $t_{1/2} < 0.5$  s). Using NMR methods that combined the use of [ $3\text{-}^{13}\text{C}$ ]-cholesterol with paramagnetic metal ions added on one side of the membrane, a  $t_{1/2}$  of  $< 2$  ms was determined for cholesterol in 30 nm SUVs composed of POPC/POPA (Bruckner et al. 2009). Although this rapid exchange may be facilitated by curvature strain in these SUVs, these experiments nevertheless demonstrate the applicability of NMR methods to resolve fast exchange processes with minimally disruptive labels. At the other end of the timescale, a  $t_{1/2}$  of 200 min has been reported for cholesterol flip-flop in POPC/cholesterol and dPOPC/cholesterol liposome preparations at  $50^\circ\text{C}$ , using small-angle neutron scattering approaches (Garg et al. 2011). Using similar SANS methodology on PC liposomes at  $37^\circ\text{C}$  containing varying quantities of cholesterol, cholesterol has been shown to slow down PC flip-flop by several orders of magnitude, from 350 min in the absence of cholesterol to  $> 4.5$  days in membranes with 40% cholesterol (Nakano et al. 2009). Under the conditions of these experiments, the PC components (DMPC or POPC) are expected to be in the fluid state at  $37^\circ\text{C}$  in the absence of cholesterol, and a liquid-ordered state at 40% cholesterol. The slower flip-flop rates are consistent with the formation of a more closely-packed

membrane with a higher  $E_a$  towards exchange. Slow flip-flop rates are more in keeping with the historical exchange rates determined for biogenic membranes such as those of enveloped viruses, using PLD or phospholipid exchange experiments, where  $t_{1/2}$  values of  $> 13$  days for cholesterol,  $> 10$  days for PC and  $> 30$  days for SM have been reported (Lenard and Rothman 1976, Rothman et al. 1976).

Recent reports of the induction of asymmetry in bilayers on solid supports (Wacklin 2011) and in liposomal membranes following the administration of poly-*L*-lysine (Brown and Conboy 2011) or the peptides melittin and alamethicin (Qian and Heller 2011), shed a note of caution on the interpretation of flip-flop rates in biogenic membranes. In thermodynamic terms, these experiments demonstrate previous theories (reviewed in Boon and Smith 2002) that the free energy benefit from the electrostatic interaction of membrane lipids with a surface or a macromolecule can be sufficient to compensate for the increase in free energy associated with the establishment of membrane asymmetry. In terms of kinetics, the asymmetry in these systems is a dynamic equilibrium that reflects different  $t_{1/2}$  values for the separate exchange processes that occur in each direction in the bilayer. To put this in a more biological context, any lipid in the cytoplasmic leaflet of a membrane that has electrostatic interactions with a membrane protein will potentially exhibit a reduced rate of exchange to the extracellular leaflet (flop) compared to that of the reverse process (flip) and as a consequence demonstrate a preferential localization in the cytoplasmic leaflet. The actual extent of asymmetry in this scenario will be determined by the free energy released by forming electrostatic interactions between the protein and the membrane lipids. The rate of flip-flop however, only depends on the magnitude of the free energy gain needed to reach the transition state,  $\Delta G^\ddagger$ . Therefore, in membranes with high asymmetry, the constituent components may still undergo fast interleaflet exchange. The ‘accessible’ component of the membrane in many studies of asymmetry, particularly for methods with low temporal resolution, may actually be reporting lipids from the extracellular leaflet *and* unbound lipids from the cytoplasmic leaflet. A particularly salient example is provided by the studies of viral envelope asymmetry described above that yield very large  $t_{1/2}$  values. As these viral envelopes are closely associated with a dense layer of matrix protein in close contact with inner surface of the envelope, it is reasonable to suspect that the reported asymmetry actually reflects interactions of specific lipids with the protein layer. From these arguments, it should be apparent that studies on flip-flop in biogenic

membranes should aim to address lipid exchange in both directions with the highest possible temporal resolution.

Taken together, the available data indicate some general patterns: flip-flop in free-standing fluid membranes generally occurs with a  $t_{1/2}$  of the order of hours to days. Flip-flop in more condensed gel phase or liquid ordered membranes tends to be slower than in fluid phases. Flip-flop in SLBs formed directly on a solid-support is faster than in the equivalent membranes when not on a solid-support, or formed on a solid support with a polymer cushion. As will be discussed below, in some cases, particularly where the membrane is stressed, lipid exchange occurs much more rapidly.

### Promotion of interleaflet exchange by peptides and proteins

Many of the studies of interleaflet exchange have been conducted using antimicrobial peptides and model peptides *in vitro*, but examples have also emerged of similar behavior with both peptides and proteins *in vivo*. The salient features of peptide-induced flip-flop are that it is both rapid and transient, occurring on a timescale of minutes following administration to the membrane (Fattal et al. 1994, Matsuzaki et al. 1996, Frascch et al. 2004). Slower translocation rates have been reported for integral transmembrane peptides (Kol et al. 2001, 2003). It should be noted that peptide-induced translocation is not a general phenomenon, particularly for transmembrane peptides (Marsh 2008). Three general mechanisms have been proposed for the increased flip-flop rates produced by peptides: (i) The formation of peptide-stabilized toroidal pores; (ii) membrane thinning induced by peptide binding; and (iii) flip-flop through membrane defects (Cho et al. 2007, Gurtovenko and Vattulainen 2007a, Anglin et al. 2009, Bocchinfuso et al. 2011, Fuertes et al. 2011, Salnikov and Bechinger 2011).

#### Toroidal pores

Peptide-induced flip-flop is frequently accompanied by poration of the membrane (i.e., release of ions or other markers), which has led to the development of models for poration that involve toroidal pores, in which the leaflets of the membrane become contiguous (Figure 3A), enabling rapid diffusive passage of lipids between leaflets. Pore formation occurs above a critical ratio of peptide to lipid (P/L<sup>\*</sup>), and as a consequence the rate of flip-flop (and pore formation) is not linear with respect to peptide concentration (Huang 2006, Fuertes et al. 2011). The nature of

toroidal pores implies that the energy barrier to flip-flop should be similar to that for diffusion in the plane of the membrane (Anglin et al. 2009), although a significant peptide density at the point of highest curvature facing the axis of the pore would be expected to hinder the rate of diffusion. Chen and co-workers used SFVS to demonstrate that administration of the peptide MSI-78 to asymmetric dDPPG/DPPG supported bilayers led to a loss of asymmetry over a time period of >10 min following administration. Their data were interpreted in terms of a toroidal pore, as peptide concentrations greater than 2  $\mu\text{M}$  were required to induce significant flip-flop (Yang et al. 2011).

#### Membrane thinning

Membrane thinning (i.e., a decrease in bilayer thickness, Figure 3B) has been demonstrated in a number of cases following the association of peptides with membranes (Lee et al. 2005, 2008, Cho et al. 2007, Marsh 2008). Thinning may arise as a direct consequence of peptide association, or as a secondary effect resulting from changes in membrane curvature. In relation to the latter, molecular modelling data suggest that membrane thinning is a consequence of increased curvature (Risselada and Marrink 2009).

Conboy and co-workers have studied the induction of flip-flop by the model transmembrane helix WALP23 and the bee venom peptide melittin by SFVS on DSPC supported lipid bilayers (Anglin et al. 2009). Representative  $t_{1/2}$  values of 195 min at 36°C and 8.6 min at 34°C were obtained for DSPC + 1 mol% WALP23 and 1 mol% melittin, respectively, against a  $t_{1/2}$  of > 1 day in the absence of any peptide. These  $t_{1/2}$  values were in accord with the corresponding free energy barriers ( $\Delta G^\ddagger$ ) for flip-flop at 37°C of 107 kJ mol<sup>-1</sup>, 103 kJ mol<sup>-1</sup> and 92 kJ mol<sup>-1</sup> for DSPC, DSPC + 1 mol% WALP23 and DSPC + 1 mol% melittin, respectively. In the presence of WALP23, the relatively small change in  $\Delta G^\ddagger$  was comprised of greater but opposing changes in  $\Delta H^\ddagger$  and  $\Delta S^\ddagger$ , with  $\Delta H^\ddagger$  being more favourable (smaller) by 30 kJ mol<sup>-1</sup>, and  $\Delta S^\ddagger$  less favourable, reflecting a more ordered transition state for flip-flop. In the case of melittin, the greater decrease in  $\Delta G^\ddagger$  for flip-flop produced by the peptide was almost entirely the result of changes in the entropic component of  $\Delta G^\ddagger$ , with the enthalpic component not changing significantly. This corresponds to a more disordered transition state for flip-flop in the presence of melittin. For both peptides, the variation in  $\Delta G^\ddagger$  was linear in respect to peptide concentration, which does not fit with a toroidal pore model for flip-flop in this system. In addition, for both peptides, the enthalpic energy

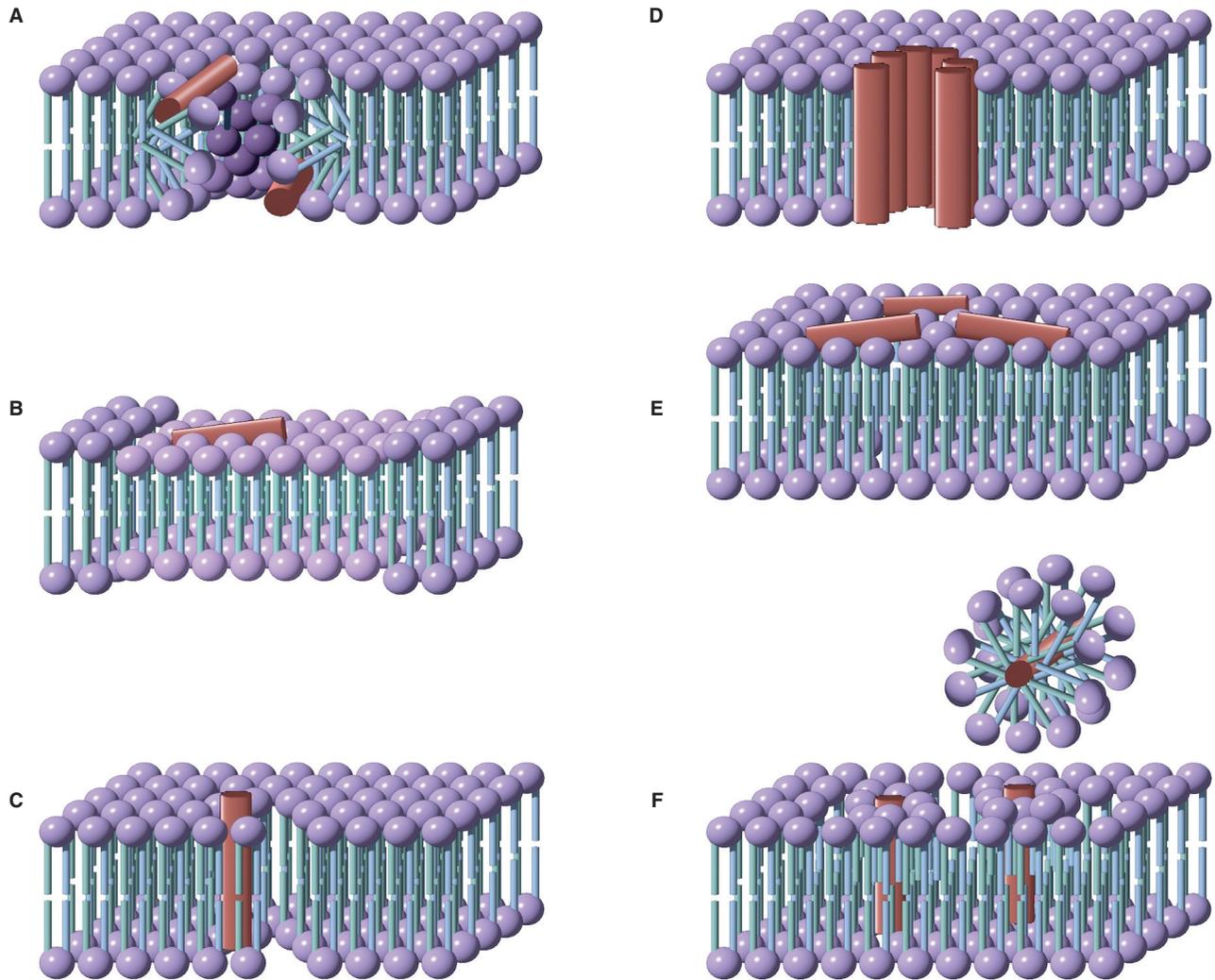


Figure 3. Models for the mode of action of antimicrobial peptides. (A) Toroidal pores, stabilized by the peptide, consisting of a lipid-lined pore and contiguous membrane leaflets; (B) Membrane thinning, in which peptide binding leads to a reduction in the thickness of the bilayer. The thinner bilayer presents a reduced barrier to the flip-flop of lipids between leaflets, as well as to the formation of transient defects; (C) Defect-mediated poration, in which the formation of transient defects (that are significantly less hydrated than toroidal pores) is promoted following peptide binding; (D) The barrel-stave model, consisting of a peptide-lined pore; (E) The carpet model, in which bilayer integrity is disrupted by the formation of peptide aggregates; (F) Detergent models, in which membrane disruption occurs through the formation of peptide-lipid micelles that remove lipids from the membrane. This Figure is reproduced in color in *Molecular Membrane Biology* online.

barrier to flip-flop was higher than would be expected for a toroidal pore. The data for melittin were therefore interpreted in terms of thinning of the membrane. These data are in accord with neutron scattering experiments that have shown that both melittin and the antimicrobial peptide alamethicin binding to the outer surface of a model membranes leads to membrane thinning *via* a reduction in thickness of the outer leaflet (Huang et al. 2004, Qian and Heller 2011). It should be noted that whilst melittin may also be implicated in the formation of toroidal pores (Yang et al. 2001, Huang 2006, Rapson et al. 2011), there have been other reports of increased flip-flop stimulated by melittin:lipid ratios below the

critical peptide to lipid ratio for pore formation, in addition to the data from Conboy (Qian and Heller 2011). Membrane thinning has been reported for non-antimicrobial peptides, including a peptide from hepatitis C virus (Cho et al. 2007).

#### *Defect-mediated poration*

A key feature of this mechanism is the creation of membrane defects following peptide binding that permit flip-flop by lower energy pathways (Figure 3C). This mechanism has been proposed for a number of peptides, including alamethicin (Pabst et al. 2007), gramicidin A (Anglin et al. 2007), and the WALP23

peptide described above (Anglin et al. 2009). As the membrane is a dynamic structure, localized defects generated by thermal fluctuations will occur spontaneously and translocation through these is likely to be responsible for the background rate of translocation in the absence of peptides or other perturbing agents (Fuertes et al. 2011). Translocation through transient pores has been observed in simulations of bilayer systems (Tieleman and Marrink 2006, Gurtovenko and Vattulainen 2007a, 2007b, Marrink et al. 2009, Gurtovenko et al. 2010, Sapay et al. 2010). The formation of transient defects following peptide binding will potentially enable sufficient penetration of the hydrophobic region of the bilayer by water to reduce the degree of headgroup desolvation required during translocation. Both the membrane-thinning and the defect-mediated poration models yield translocation rates that increase approximately linearly in respect of peptide concentration. In many respects there is a large degree of synergy between the defect-mediated and membrane-thinning models. Association of molecules with the membrane interface in one leaflet of the bilayer is sufficient to increase the area of this leaflet, the ramifications of which are membrane thinning and a difference in surface tension between the leaflets that reduces the barrier to thermally-driven pore formation (Ludtke et al. 1995, Longo et al. 1998, Lee et al. 2008, Wimley 2010, Qian and Heller 2011). In common with the membrane thinning model, in defect-mediated poration the barrier to flip-flop is reduced, but the latter model is distinguished by more localized lipid disorder. The defect-mediated model may be further differentiated from both the toroidal pore and thinning models in having the faster apparent kinetics, primarily because membrane disorder is the immediate consequence of peptide binding. Added to this, solvent penetration of the bilayer, either following peptide-membrane binding or peptide desorption from the membrane (as peptide-membrane binding is intrinsically reversible), is expected to occur faster than lipid restructuring to anneal defects and lateral peptide diffusion during to drive assembly into toroidal pores. As a consequence, it is to be expected that in some experiments, complex translocation kinetics will be observed.

Other forms of membrane perturbation enhance the rate of translocation, including the process of heating or cooling a membrane through the gel to liquid crystalline phase transition (De Kruijff and Van Zoelen 1978). It is also of note that in both the paper by Kornberg and McConnell, and more recent studies (Kotova et al. 2011, Volinsky et al. 2011), the presence of oxidized lipids has been found to accelerate transbilayer exchange of membrane lipids. In both of the latter cases, packing defects generated by

the oxidized lipids were proposed as the principal reason for translocation, although there are differences in the extent of water penetration of these defects in the two cases, ranging from little penetration (Kotova et al. 2011) to water-filled pores spanning both leaflets (Volinsky et al. 2011). Interestingly, when photodynamic methods are used to generate oxidized lipids (Kotova et al. 2011), the defects produced are sufficient to enable the passage of small organic molecules, but not ions, which is consistent with a sparsely hydrated pore. Most membranes *in vivo* are characterized by the existence of an electrochemical potential gradient, which may serve to drive the formation of more long-lived pores from transient defects (Gurtovenko and Vattulainen 2005, 2007a, 2007b, Gurtovenko et al. 2010). Whether a pore extending across the entirety of the bilayer is required for lipid translocation is not certain; partial penetration of the hydrophobic interior of the bilayer by water will lead to similar effects to membrane thinning in terms of translocation rates.

## Peptide-membrane binding kinetics

### *Models for peptide-membrane binding*

A vast body of work has accrued on the subject of peptide interactions with membranes, much of it associated with understanding the mechanisms by which antimicrobial peptides operate. Models for antimicrobial peptide activity have been well reviewed (Huang et al. 2004, Reddy et al. 2004, Bechinger and Lohner 2006, Huang 2006, Shai 1999, Epand and Epand 2009, Wimley 2010). Salient models are summarized in Figure 3. Historically, general models such as the barrel stave, toroidal pore and carpet models, as well as those involving detergent activity, have been peptide-centric, focused to a large degree on the peptide structures that form at equilibrium. In considering the process of peptide-membrane binding, understanding the kinetics of each stage of the process is essential, both with regard to the time that is required for equilibrium to be attained, and the timing of events such as marker release in relation to the equilibration process (Schwarz et al. 1987). Marker release following the administration of an active peptide is usually rapid and frequently involves biexponential kinetics, with the fast initial release on a timescale of seconds followed by a slower phase on a timescale of minutes to hours (Schwarz and Robert 1990, 1992, Mazzuca et al. 2010). In order to relate marker release kinetics to the binding process, it is necessary to consider a holistic approach that involves explicitly monitoring of the kinetics of *both* peptide *and* lipid processes within the mixture. Studies of this

nature are infrequent; the majority of cases focus measurements on either peptide or lipid properties, with phenomena such as marker release usually implicitly interpreted in terms of peptide models. Recently, a more lipid-centric viewpoint has been called for that is more in keeping with the kinetics of poration phenomena (Fuertes et al. 2011). Before considering more lipid inclusive models, it is useful to consider alternative models for interpreting data from marker release experiments.

#### *All-or-none or graded release*

A fundamental issue with regard to antimicrobial peptide models concerns identification of the equilibrium structures that form. One of the methods that may be used to distinguish mechanisms that involve a discrete pore (barrel stave and toroidal pore) from those that involve transient poration (detergent-based and carpet model) is the nature of marker release observed from lipid vesicles containing entrapped markers (Schwarz and Robert 1990, 1992, Ladokhin et al. 1997, Arbuzova and Schwarz 1999). For all-or-none release, the formation of a stable long-lived pore at concentrations greater than a threshold concentration ( $P/L^*$ ) leads to rapid and complete release of entrapped markers from individual vesicles. The sample then comprises a mixture of completely empty vesicles and completely filled vesicles. Furthermore, the addition of a new marker to the solution outside the vesicles leads to marker ingress into porated vesicles only (Gregory et al. 2008, 2009).

By contrast, graded release is characterized by a slow release of markers over a finite time window following addition of the peptide, with the timescale for cessation of net marker release typically of the order of several minutes at non-saturating peptide concentrations (Pokorny and Almeida 2004, Gregory et al. 2009, Apellániz et al. 2010). The sample at this point comprises vesicles with varying states of partial emptiness. Addition of a new marker outside the vesicles does not lead to partial marker ingress into all vesicles. The recent wide adoption of giant unilamellar vesicles (GUVs) as model vesicular systems, combined with confocal microscopy, has provided some salient examples of all-or-none and graded poration. These experiments reveal details of the rate of marker influx or efflux once a long-lived pore has formed, as well as the time required for pore formation to occur. For example, two peptides from the HIV gp41 fusion protein were shown to differ in their mode of action in an assay that measured the rate of fluorescent marker influx into GUVs simultaneously treated with a peptide and the marker (Apellániz et al. 2010). One of the peptides, termed

CpreTM, induced all-or-none poration of GUVs, characterized by fast filling kinetics, with GUVs reaching 80% filled within  $\sim 100$ s. However, this rate of marker ingress was only achieved once a stable pore had formed. The rate of pore formation, as determined by the lag between adding the peptide and marker ingress starting, varied between 0 and 20 min across the sample. By contrast, the other peptide, termed NpreTM, exhibited a graded mechanism, with the extent of filling not reaching 30% after 30 h of incubation. This peptide also displayed a lag phase between peptide addition and poration commencing. Similar lag phases have been reported for marker release experiments using GUVs (Vad et al. 2010). Treatment of GUVs with the peptide Bax $\alpha$ 5 led to the formation of stable pores, with some vesicles remaining permeable to markers after several hours, consistent with an all-or-none mechanism (Fuertes et al. 2010). Pores formed over longer time scales were smaller than the pores formed after initial peptide binding, suggesting that a rapid formation of non-equilibrium pores occurred alongside assembly to form stable pores. The differences between all-or-none and graded release revealed by GUV experiments may generally be interpreted in terms of equilibrium and non-equilibrium structures, as will be described below, provided that both peptide and lipid processes are accounted for.

#### *A more general model for peptide-lipid interactions*

In order to account for peptide and protein association with membranes in a lipid-inclusive manner, a more general model is presented below (Figure 4). The key to this model is that any change in the nature of the interaction between a peptide and a membrane, whether this involves a structural change in the peptide, peptide reorientation within the membrane, or peptide oligomerization, perturbs the bilayer structure and is therefore accompanied by a corresponding process of lipid relaxation towards an intermediate equilibrium state. Initial association with the membrane produces a state (A) in which neither the peptide nor the lipid component are at equilibrium. The membrane in this state is likely to be the highly prone to defects as it is the furthest from equilibrium. The initial membrane-bound state then relaxes to an intermediate membrane bound form (B). The equilibrium between states A and B can be considered as a direct process (with forward rate constant  $k_1$ ) or as microscopic equilibria for lipid and peptide relaxation with forward rate constants of  $k_{1L}/k_{1L}$  and  $k_{1P}/k_{1P}$  respectively. The intermediate state (B) then undergoes further transformation, such as peptide realignment, with similar lipid relaxation to give the next

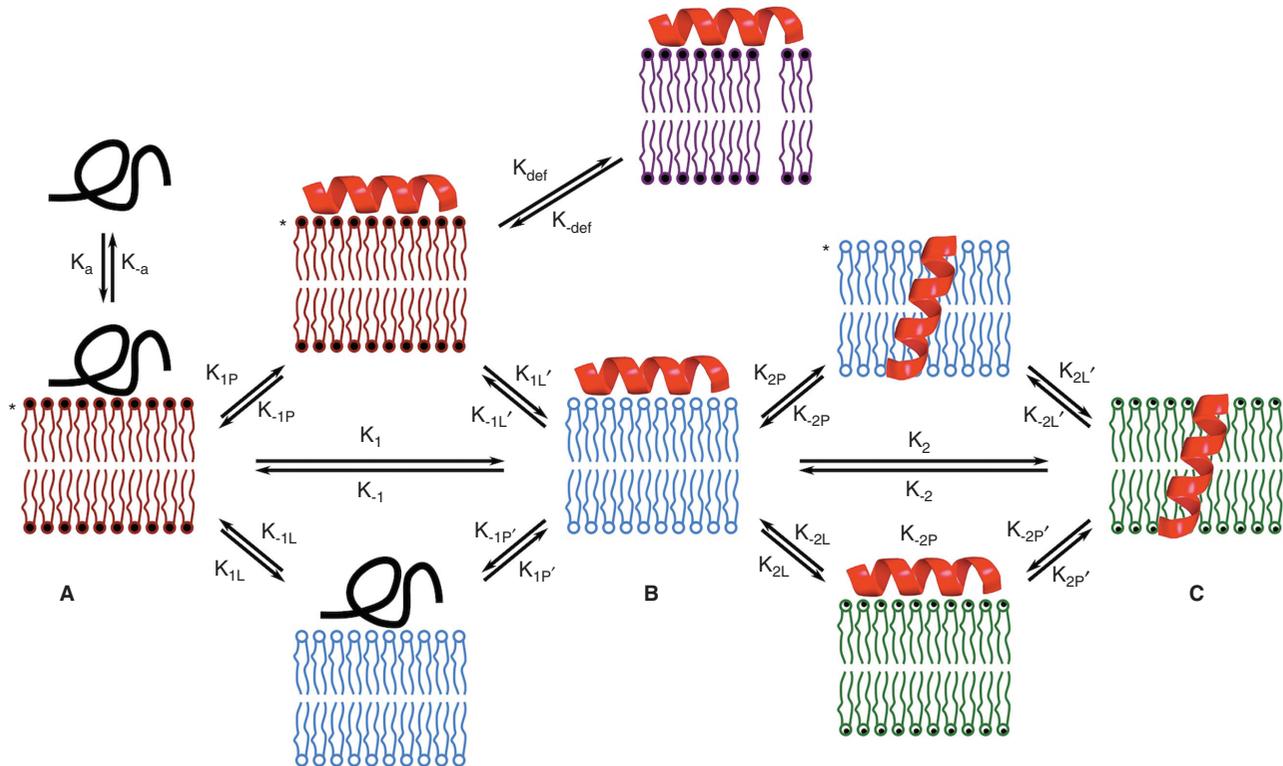


Figure 4. A model for peptide-lipid interactions that incorporates the kinetics of both peptide and lipid-based transformations. Forward processes involving peptides and lipids have 'P' and 'L' subscripts respectively. Intermediate *A* is formed immediately following peptide binding. As shown here, the unfolded peptide initially binds, as occurs in many (but not all) cases. Adoption of peptide 2° structure leads to intermediate *B* following lipid relaxation. Intermediate *B* in many systems corresponds to a bilayer of reduced thickness following thinning. Intermediate *C* could correspond to the system following peptide insertion (as shown), but in a general scheme *C* would correspond to any system that involves a peptide rearrangement from *B*, followed by lipid relaxation. Processes such as defect formation (represented by  $k_{def}$ ) will be favored in those intermediates (indicated by asterisks) in which the lipid component of the system is not at equilibrium. This Figure is reproduced in color in *Molecular Membrane Biology* online.

intermediate state (*C*) and so on. All states are potential precursors for thermally-induced transient defects, but the states in which the membrane component is in a more disordered (non-relaxed) state (indicated by asterisks in Figure 4) will lead to enhanced defect formation. This model is imperfect, particularly as it does not allow for co-operative interactions between peptide and lipid, and parallel pathways for the formation of peptide states, but it is nevertheless useful for addressing the outcomes of a number of peptide-membrane binding experiments.

#### Initial association with the membrane

Fluorescence methods have been widely used to monitor the association of peptides with membranes. Recently, total internal reflection fluorescence (TIRF) imaging has been used to monitor the binding and unbinding of single glucagon-like peptide-1 molecules from a supported bilayer surface, leading to respective values for  $k_a$  and  $k_{-a}$  of  $1.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$

and  $8.2 \times 10^{-1} \text{ s}^{-1}$  (Fox et al. 2009). An additional feature of this method is the ability to track individual peptides on the bilayer surface, giving access not only to the residence time of individual peptides (which reflects the bound state of the peptide in the membrane), but also lateral diffusion coefficients.

The intrinsic fluorescence of native tryptophan residues in the sequence of a protein provides a convenient tool for monitoring both initial binding to the membrane, and subsequent equilibria following binding. In general, initial peptide binding to the membrane is accompanied by increases in Trp fluorescence intensity and a blue shift in the emission maximum (Ladokhin and White 2004). For some proteins however, unexpected changes in Trp fluorescence can reveal details not accessible by other means, such as partial protein unfolding during the fast initial binding step, exemplified by red shifted emission maxima and increased fluorescence intensities following the binding of chicken liver bile acid-binding protein to negatively-charged membranes (Galassi et al. 2009). The same article additionally demonstrates that a

single Trp residue is sufficient for experiments to monitor either intrinsic Trp fluorescence intensity, Trp as a FRET donor to NBD, or quenching of Trp fluorescence by acrylamide. The suitability of Trp as a FRET donor has also been exploited in stopped flow experiments to monitor the association of an analog of the antimicrobial peptide magainin 2 with membranes composed of POPC/POPG. This yielded a value for  $k_a$  of  $7.8 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$  and a value for  $k_{-a}$  of  $88 \text{ s}^{-1}$  for POPC/POPG (4:1), with  $k_a$  increasing with increasing PG composition of the membrane, and  $k_{-a}$  decreasing exponentially (Gregory et al. 2009).

Two methods are particularly useful for measuring the rate at which peptides adsorb and desorb from the membrane surface: surface plasmon resonance (SPR) (Mozsolits and Aguilar 2002, Papo and Shai 2003, Kamimori et al. 2005, El Amri et al. 2006) and analysis using a quartz crystal microbalance (QCM) (Wacklin 2011). Although these measure only the total quantity of peptide associated with the membrane, their temporal resolution is such that  $k_a$  and  $k_{-a}$  can be measured accurately, allowing the association constant to be determined as  $k_a/k_{-a}$ . For example the kinetics of binding of the protein annexin A1 to a supported bilayer with POPC/POPS (4:1) as the distal (solution-facing) leaflet were determined using a QCM, which enabled values for  $k_a = 53 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  and  $k_{-a} = 7 \times 10^3 \text{ s}^{-1}$  to be determined in the presence of  $0.1 \text{ mM Ca}^{2+}$  (Kastl et al. 2002). The inclusion of cholesterol in this system did not significantly alter the association and dissociation constants. Related experiments with annexin A2t yielded data of a similar magnitude ( $k_a = 34 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$  and  $k_{-a} = 1.4 \times 10^3 \text{ s}^{-1}$ ) (Ross et al. 2003). The rate of membrane association of the bovine seminal plasma protein PDC-109 as a function of temperature and the lipid composition of supported bilayers containing 20 wt% cholesterol on a gold surface were investigated by SPR. This method gave access to both  $k_a$  and  $k_{-a}$  values. Typical values for  $k_a$  at  $20^\circ\text{C}$  were  $5.7 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ,  $1.2 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$  and  $5.3 \times 10^2 \text{ M}^{-1} \text{ s}^{-1}$  for surface layers composed of DMPC, DMPA and DMPG respectively (Thomas et al. 2003). Typical respective values for  $k_{-a}$  at  $20^\circ\text{C}$  were  $2.7 \times 10^2 \text{ s}^{-1}$ ,  $11.0 \times 10^2 \text{ s}^{-1}$  and  $9.0 \times 10^2 \text{ s}^{-1}$  for the same surface layers. The kinetics of membrane association of the C2 domain of protein kinase C $\alpha$  (PKC $\alpha$ ) were found to more complex than a simple 1:1 model by SPR, but nevertheless it was apparent that the half-life for equilibration of binding was of the order of 50 s for surface layers containing PS or PI. A higher overall affinity for surface layers containing PI was reflected in a slower rate of desorption ( $k_{-a}$ ) in this case (Manna et al. 2008). SPR has equally been

applied to follow the kinetics of peptide binding (Kamimori et al. 2005, Zhang et al. 2000).

A general feature of the kinetics described above is that  $k_a$  is generally slower than the diffusion-controlled rate for reactions ( $\sim 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ ) (Isaacs 1995) by several orders of magnitude, which implies that there is a free energy barrier to binding (Fox et al. 2009). In the case of the SPR experiments with PDC-109 (Thomas et al. 2003) described above, the differences in  $k_a$  values were largely accounted for by entropic changes in the transition state for binding, with a positive (favourable)  $\Delta S^\ddagger$  for DMPC and a negative  $\Delta S^\ddagger$  for the other lipids.

#### *Marker release experiments*

A key question in all marker release experiments is the underlying mechanism by which membrane permeation occurs. This can be expressed in terms of whether a peptide-stabilized ensemble is formed, as in the toroidal pore, barrel stave, or carpet mechanisms, or whether permeation is a consequence of changes in bilayer stability that lead to increases in the occurrence and lifetime of transient thermally-induced defects. A fundamental issue is whether marker release kinetics are reporting peptide association with the membrane ( $k_a$ ) or the rate of processes that occur following association, including peptide reorganization ( $k_{1P}$ ,  $k_{1P'}$ ) and lipid relaxation ( $k_{1L}$ ,  $k_{1L'}$ ) or a combination of both ( $k_1$ ). A key requirement is identification of the rate determining step for marker release, which may be any of these processes, as well as the rate of marker diffusion along the permeation pathway ( $k_{\text{efflux}}$ ). The distinction between stable protein ensembles and efflux mediated by transient non-equilibrium structures can be made by assessing whether marker release is graded or all-or-none using fluorescence-based approaches (Ladokhin et al. 1997, Pokorny and Almeida 2004). Recently the kinetics of marker release have been used to probe the poration mechanism, with the data modeled as an initial peptide binding step followed by a transformation to a permeative state (Gregory et al. 2008, 2009). The kinetics of peptide association and dissociation were determined independently of marker release, and the rate of marker permeation ( $k_{\text{efflux}}$ ) was accounted for, bringing this method close to model in Figure 4 without direct consideration of membrane equilibration. No assumptions were made concerning the nature of the permeative state, although a model that included peptide oligomerization did not fit the data well. Using this approach, cecropin A could be shown to operate with an all-or-none mechanism in POPC/POPG membranes, whereas analogs of

magainin 2 changed from an all-or-none mechanism to a graded mechanism as the PG content of POPC/POPG membranes fell below  $\sim 30$  mol%. It is notable that the kinetics of marker release in these experiments were fast, with  $t_{1/2}$  typically  $< 1$  min for cecropin A with membranes composed of POPC/POPG (1:1), and  $\sim 6$  min for POPC/POPG (4:1). Equivalent values for magainin 2 were  $t_{1/2} \sim 4$  min and  $\sim 20$  min, respectively, in the same lipid systems. A rigorous study has addressed marker release induced by the peptide pardaxin from liposomes composed of either DOPC or DOPC/DOPG (4:1) containing calcein as the marker (Vad et al. 2010). The data demonstrate complex biphasic marker release kinetics in response to changes of pH and peptide concentration. For DOPC, marker release occurred more rapidly with increasing pH and reached a saturating value of  $t_{1/2} = 0.02$  min at pH 10 for a P/L of  $\approx 0.0002$  for the fast phase, and  $t_{1/2} = 0.2$  min at pH 10 for a P/L of  $\approx 0.0005$  for the slow phase. At the same P/L at pH 5.5, the  $t_{1/2}$  for the slow phase increases to several minutes. For DOPC/DOPG, the rates of marker release were fastest at pH 5.5, with values for  $t_{1/2}$  of 0.01 min (P/L  $\approx 0.005$ ) and 0.07 min (P/L  $\approx 0.01$ ) being typical for the fast and slow phases, respectively. Further experiments were conducted by  $^{13}\text{C}$  NMR, which indicated that the peptide inserts into a transmembrane arrangement in DOPC liposomes, but remains peripherally bound in membranes containing DOPG. The marker efflux kinetics for pardaxin can be accounted for by complementarity between the net charge of the peptide and the membrane at specific pH values, and differences in peptide rearrangement that occur following initial membrane association. The examples above illustrate that the processes which lead to marker efflux are inherently rapid for both defect-mediated poration and peptide-mediated poration where the peptide remains bound peripherally, which is in accord with the in-plane diffusion coefficients ( $D_{\parallel}$ ) for lipids and proteins, which are the same order of magnitude in model systems where both are addressed (although peptides and proteins diffuse more slowly than lipids in accordance with their higher molecular weight). The kinetics of peptide insertion to transmembrane configurations are more varied and are discussed in more detail in the next section.

In many cases, marker release is accompanied by translocation of lipids between leaflets, with both processes occurring on a similar timescale following peptide administration (Arbuzova and Schwarz 1999, Pokorny and Almeida 2004, Fuertes et al. 2011, Kotova et al. 2011). The kinetics data therefore suggest that rapid membrane poration and lipid

translocation share a common mechanism. Mechanisms that involve increased lipid disorder, whether directly as a response to local perturbation following peptide binding (Fernandez et al. 2011), or as a consequence of membrane thinning, are good candidates. The formation of transient defects that enable significant water ingress into the hydrophobic interior of the bilayer will benefit both lipid translocation and membrane permeation in the same manner by reducing the requirements for desolvation.

### Changes in peptide-membrane systems occurring over longer time scales

In many of the cases discussed above, initial rapid change of the experimental parameter being monitored is followed by one or more slower phases. Slow kinetic processes are typically observed in situations that involve peptide insertion to transmembrane arrangements, such as the case for pardaxin in DOPC membranes (Vad et al. 2010). In terms of the model in Figure 4, this corresponds to  $k_{P2}$  and  $k_{P2'}$ , as well as the corresponding lipid relaxation processes ( $k_{L2}$  and  $k_{L2'}$ ). Transmembrane insertion is inherently sensitive to complementarity between the net charges of the peptide and the membrane, which is apparent in the case of pardaxin association with neutral DOPC described above, where decreasing the pH increases the net charge on the peptide, with concomitant decreases in the rate of peptide insertion and marker efflux. Insertion of the model peptide TMX-3, when monitored by intrinsic Trp fluorescence, similarly exhibits significantly slower insertion kinetics at pH 5 ( $> 60$  min) when compared with pH 6.3 ( $\sim 1$  min) (Ladokhin and White 2004).

Direct measurements of peptide insertion that follow changes in peptide spectral properties, such as intrinsic Trp fluorescence in the example above, are a robust means of quantifying slow kinetic processes. Linear dichroism (LD) spectroscopy and oriented circular dichroism spectroscopy (OCD) similarly provide direct measurements of peptide behavior in membranes, both yielding data that reveal the orientation of absorbing chromophores. When applied to studying the membrane association of peptides, the predominant chromophores arise from the peptide backbone and aromatic side chains, as lipids have extremely weak chromophores. OCD is closely related to conventional CD spectroscopy, with the difference being that the membranes are aligned with respect to the experimental frame (Wu et al. 1990, Yang et al. 2001, Chen et al. 2003, Lee et al. 2004, 2005, Huang 2006, Bürck et al. 2008, Qian et al. 2008, Cheng et al. 2010). LD spectroscopy measures the differential absorbance by the sample of linear and

parallel polarized light (Rodger et al. 2002, Castanho et al. 2003, Caesar et al. 2006, Esbjörner et al. 2007, Svensson et al. 2011). An aligned sample is an absolute requirement for LD: non-aligned samples do not yield an LD spectrum. Alignment is usually achieved using bilayers adsorbed in glass plates, or by shear flow (Dafforn et al. 2004, Marrington et al. 2005). Changes in the alignment of a peptide with regard to the membrane can be monitored by both LD and OCD in real time. LD spectroscopy has the additional advantage that peptides not associated with the membrane are not aligned and therefore do not contribute to the observed signal. In these experiments, only changes in peptide orientation are monitored; as a consequence the data are interpreted in terms of  $k_1/k_{-1}$  and  $k_2/k_{-2}$  (Figure 4). Studies by OCD and LD can detect the presence of multiple peptide equilibria and have revealed that models that involve equilibria between peripheral and membrane inserted states are accurate for many systems (Ennaceur et al. 2009, Svensson et al. 2011). It is apparent from these experiments that complete equilibration of the peptide-lipid system is only achieved after significant periods of time. For example, association of the peptide melittin with DMPC membranes yielded complex behavior when studied by LD, requiring >7 h for equilibration at 25°C and ~ 10 min at 37°C (Damianoglou et al. 2010). Similar periods of hours were required for complete equilibration in POPC/DOPG (4:1) membranes (Svensson et al. 2011). Model peptides have similarly yielded slow rates for  $k_2$ , attributed to membrane insertion on the basis of the LD data, with a  $t_{1/2}$  of 75–100 min (Ennaceur et al. 2009).

Esbjörner et al. (2007) studied the association of the HA2 fusion peptide from influenza virus hemagglutinin with synthetic membranes composed of POPC or POPC/POPG (4:1) using a range of methods, including LD and intrinsic fluorescence. The latter approach further enabled the fast association with the membrane ( $k_a$ ) to be resolved, with a  $t_{1/2}$  of < 2 s. Interestingly, marker release experiments revealed membrane poration only during this fast kinetic phase, with marker release more prominent from POPC vesicles. A second, slower phase was detectable by both intrinsic fluorescence and LD, with a  $t_{1/2}$  > 1 h. A key feature of this work was the use of membrane-embedded retinoic acid as an LD marker for membrane order. This enabled the kinetics of membrane relaxation to be followed in tandem with changes in peptide orientation. Two fundamental observations resulted: Firstly, the extent of marker release in the fast phase correlated with the degree of membrane disorder in this phase; secondly equilibration of the LD signal from retinoic acid required

periods in excess of 1 h, indicating that membrane relaxation in this system is slow and potentially coupled to protein reorientation. NMR relaxation experiments have demonstrated that even in the equilibrium state, there can still be significant peptide rocking motions of hemagglutinin with respect to the membrane (Lorieau et al. 2011), which acts as a salient reminder that spectroscopic methods such as fluorescence and LD measure the average properties of the species in question.

It has recently become evident that even in simple model peptide-lipid systems, the lipid cannot be considered as an inert medium in which peptide binding and reorientation occurs, with acyl transfer from the lipid to the peptide melittin from POPC membranes, in the absence of enzyme catalysis, detectable as a background reaction with a  $t_{1/2}$  of ≈ 16 days in phosphate buffered saline (Pridmore et al. 2011). The same reaction has a  $t_{1/2}$  of ≈ 24 h in bicarbonate buffer at pH 7.4, with acylation products detectable after 4 h (Dods et al. 2012). Acylation occurs primarily at the N-terminal amino group of melittin, as well as the amino groups of lysine side chains, most notably the lysine closest to the C-terminus of the peptide. Traces of doubly-acylated peptide, alongside putative acyl transfer to the side chain of a serine, could also be detected. The extent to which this reactivity is typical for membrane-active peptides and proteins is yet to be established. Anomalous behavior observed with other peptides, such as TMX-3, which displayed irreversible membrane binding (Ladokhin and White 2004), may also be the consequence of peptide acylation by lipids, although this is currently unproven.

#### *In vivo studies that address both early and late stages of binding*

Studies on the association of peptides with cell membranes *in vivo* generally reveal that fast processes such as membrane depolarization occur on a timescale that is slower than the  $k_a$  in model systems, but nevertheless still sufficiently rapid that  $t_{1/2}$  values are < 10 min. <sup>125</sup>I-labeled streptolysin O exhibited biphasic binding kinetics with red blood cell membranes, with a fast initial binding ( $t_{1/2}$  ~ 70 s) at 37°C followed by a slower phase with a timescale of ( $t_{1/2}$  ~ 10 min) during which streptolysin O oligomers assembled (Palmer et al. 1995). The binding of a number of peptides to *Staphylococcus aureus* membranes was assayed by addressing the rate of membrane depolarization and survival rate following peptide administration. In general, depolarization occurred with a  $t_{1/2}$  of 2–3 min, with cell death requiring longer time frames (tens of minutes) (Friedrich et al. 2000). A similar  $t_{1/2}$

for membrane depolarization was observed following the administration of aurein analogs to *S. aureus* C622 (Cheng et al. 2010). Addition of melittin to sheep lymphocytes loaded with calcein yielded distinct biphasic marker release kinetics, with a fast initial release of marker ( $t_{1/2} \sim 1$  min) and a much slower second phase ( $t_{1/2} \sim 1$  h) (Su et al. 2001).

Recent improvements in atomic force microscopy have enabled individual images to be obtained within a time span of 10–15 s or less, making high-speed AFM a viable tool for studying peptide- and protein-membrane binding. This has enabled the effects of administration of the antimicrobial peptide CM15 to *Escherichia coli* to be monitored in real time (Fantner et al. 2010). Noticeable roughening of the surface of the bacterium was detectable within 2 min of administration of the peptide, consistent with a rapid initial adsorption of the peptide ( $t_{1/2} = 52 \pm 16$  s) and a  $t_{1/2}$  for bulk killing of 4.6 min.

### Summary of peptide binding kinetics

From the arguments presented above, some general features of the association of peptides and proteins with lipid membranes can be described. The initial rate of association ( $k_a$ ) is fast ( $t_{1/2}$  of milliseconds to seconds) and 1 to 5 orders of magnitude slower than the diffusion of water. Peptide dissociation from the membrane ( $k_d$ ) is generally 3 to 6 orders of magnitude slower than association, depending on the strength of the association with the membrane. Following peptide association, both initial structural changes to the peptide, and membrane reorganization are fast ( $t_{1/2}$  of milliseconds to seconds), and assembly to permeative states also fast ( $t_{1/2}$  of seconds). Rates of subsequent processes, particularly membrane reorientation (i.e., insertion to transmembrane conformations) yield much greater variations in rate profiles, with  $t_{1/2}$  values ranging from seconds to hours.

### Conclusions

The data obtained from model systems have demonstrated the fundamental properties of lateral, transverse and rotational diffusion in membranes. Nevertheless, a number of recent observations have fuelled debates that have implications for our understanding of both model and biogenic systems. One of the foremost issues concerns the structure of SLBs. A better understanding of the structure of SLBs at the molecular level will be central to comprehending the atypical properties exhibited by some SLBs, such as fast transverse diffusion and electrostatically-induced asymmetry. Biogenic membranes exhibit more diverse behavior than their model counterparts, largely as a

consequence of a high protein content, more complex lipid composition, and interactions with the cytoskeleton. Whilst molecular diffusion in biogenic membranes is generally slower than in model systems, anomalous diffusion in biological systems is widely reported, manifested by localized variations in diffusion rates. Understanding why phenomena such as anomalous or confined diffusion are specific to certain membranes or induced by under specific conditions will lead to a better understanding of the kinetics of membrane processes such as receptor clustering and membrane remodelling. The role of cholesterol in moderating membrane properties continues to provoke much argument. This is reflected both by the large diversity of reported transverse diffusion rates and the inability to observe the macroscopic phase separation *in vivo* that is seen *in vitro*. Improvements to the methods available for studying the lateral diffusion of membrane components in biological membranes, such as FCS-STED and NMR, will enhance our understanding of how cholesterol interactions with specific lipids classes lead to lateral asymmetry and the formation of rafts, as well as the more general role of cholesterol in moderating membrane fluidity.

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