Abstract: In solid-state NMR of biomolecules, the average structure and dynamics are addressed by combining experimental results with theory. Relaxation rates exhibit a functional dependence on order parameters because of molecular motions and/or collective excitations of liquid-crystalline membranes. Mixtures of phospholipids with cholesterol or nonionic surfactants allow the experimental correspondence of $^2$H NMR observables to be quantitatively tested. For cholesterol-stiffened bilayers, the spin-lattice relaxation rate profile is reduced together with an increased order profile. Bilayer softening due to nonionic surfactants gives an opposite relaxation enhancement accompanied by reduced order parameters. In both cases a square-law functional dependence (Fermi's Golden Rule) explains the relaxation and order profiles in terms of mean-square amplitudes of the lipid fluctuations. Model-free analysis reveals an $\omega^{-1/2}$ frequency law for three-dimensional (3D) fluctuations of the membrane, whereas for two-dimensional (2D) elastic sheets an $\omega^{-1}$ dependence is expected. Collective segmental or molecular modes emerge on the mesoscale of the bilayer thickness and smaller that are formulated with continuum elastic theory. Furthermore, the bilayer core resembles a hydrocarbon fluid with a viscosity of only a few centipoises (cP). Magnetic resonance spectroscopy thus reveals properties of the membrane lipids described by a hierarchical energy landscape that affects their polymorphism, phase behavior, and lipid-protein interactions.

Key words: cholesterol, director fluctuations, elastic modulus, molecular dynamics, solid-state NMR, spin-label EPR, order parameter, lipid rafts, relaxation

7.1 Introduction

Nuclear magnetic resonance (NMR) spectroscopy offers one of the premiere biophysical tools for addressing the structural and dynamical properties of biomolecules, including proteins, lipids, and nucleic acids. The spectral lineshapes give us knowledge of the average molecular structure in analogy to X-ray or neutron diffraction, while the nuclear spin relaxation times manifest the fluctuations around the mean conformation.

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Together, the solid-state NMR spectral lineshapes and relaxation rates address the problem of disentangling the mean-square amplitudes and rates of the motions versus the forces and potentials that govern the molecular properties. For membrane lipids, the dynamics can include local segmental motions, reorientations of the entangled molecules, and harmonic or elastic excitations of the bilayer [1, 2]. An important question is whether the fluctuations are mainly local, or whether the dynamics are inherently collective and entail a hierarchical energy landscape [1, 3–5]. Here we furnish a perspective of how information of biochemical or biomedical significance is captured in relation to molecular mechanisms and functions of membranous soft matter [6].

In applying magnetic resonance spectroscopy (spin-label electron paramagnetic resonance, EPR, and NMR) to biomolecules the study of lipid membranes is particularly illuminating [5, 7–16]. Proteins and lipids exist within a fluid bilayer matrix that shares many features in common with the liquid-crystalline state [1, 17–19]. A comprehensive view considers both the ordering and dynamics of the molecules [1, 20, 21]. Investigations of phospholipids, cholesterol, peptides, and membrane proteins address their average structures and molecular dynamics within the membrane [21–26] – multiple scales of time and space are involved. Such multi-scale approaches to solid-state NMR spectral lineshape measurements and relaxation times can be uniquely instructive [27]. The convergence of advances in NMR technology with biophysical knowledge emphasizes some of the most challenging and timely questions in contemporary membrane science [6, 28–31]. Combining solid-state NMR spectroscopy with relaxation measurements allows one to investigate the collective dynamics of membrane liquid crystals, in which a unified view of the relaxation frequency (magnetic field) and order parameter dependence suggests the emergence of bilayer elasticity at an atomistic level. Nuclear spin relaxation studies [32] together with spin-label EPR results [3, 5] and complementary scattering approaches [33–37] thus inform the emergent biophysical properties of cellular membranes.

### 7.2 A brief history of membrane biophysics

Some readers may think the subject of lipid bilayer dynamics – much less biomembranes – is far too complicated to be understood at any semi-rigorous level of physics or chemistry. Perhaps it is best left to the cell biologists. How can such a messy and disorderly biological system with countless degrees of freedom, and with flexible chain molecules that undergo all sorts of internal isomerizations, as well as rotational and lateral diffusion, be possibly understood at any level of rigor, for example, as in other areas of physics or chemistry [38]? We would submit that the answer is: yes. Below we justify this assertion, which rests on a confluence of molecular spectroscopy with comparative thinking about the properties of molecular liquids, solids, and liquid crystals.
7.2.1 X-ray scattering and magnetic resonance spectroscopy

Lipid bilayers are central to structural biology because they are the stuff that cellular membranes are made of. Various proteins exist in contact with the lipid bilayer, beginning from their birth on the endoplasmic reticulum, and ending up with the new membrane material. Because lipid bilayers encapsulate the features of soft matter [6], understanding their dynamics is vital to biophysics (see Figure 7.1). Levine and Wilkins [39] recognized this aspect very early on using X-ray diffraction, and pointed out the liquid-like interior of the lipid bilayer [39]. Yet X-ray scattering [37, 40–42] is handicapped by its utility mainly as a structural tool. By contrast, magnetic resonance spectroscopy (spin-label EPR and NMR) provides site-specific knowledge of both the structure and dynamics [2, 32]. In a series of early papers, Chapman et al. [43, 44] used proton NMR methods (primitive by today’s standards) to investigate the gel and liquid crystal states of membrane lipids, thus providing further evidence for a fluid-like bilayer. Current magnetic resonance applications follow these leads, and seek to explain how membrane structure, dynamics, and function are related.

![Figure 7.1](image)

**Figure 7.1:** Magnetic resonance spectroscopy (NMR and spin-label EPR) explores the hierarchical energy landscapes and fluctuations of lipid membranes. The dynamics include segmental motions, molecular diffusion, and viscoelastic deformation. Fluctuations involve the geometry of the interactions via Euler angles (Ω) for transformations between various coordinate frames, together with the mean-square amplitudes and correlation times (τC) of the motions. Coordinate frames are designated as follows: I, intermediate (segmental) frame; M, molecular interaction frame; N, local director frame; and D, bilayer director frame. The multi-scale dynamics of membranous soft matter involve a broad range of time and length scales. Figure adapted with permission from Ref Leftin and Brown [32].
However, perhaps the singular development that established the fluid-like nature of phospholipids was spin-label EPR spectroscopy, as originally introduced by Wayne Hubbell and Harden McConnell at Stanford [7]. In a series of innovations, they produced not only the first nitroxide spin probes of phospholipids, but also worked out the basic resonance theory as put forth in the classic text of Carrington and McLachlan [45]. Closely related work by Joachim Seelig for membrane liquid crystals [46] was likewise seminal in establishing the flexibility gradient of the lipid chains of membrane bilayers. And in more or less concurrent proton NMR studies, Sunney Chan and his students at Caltech discovered site-specific differences in relaxation times of membrane lipids [47, 48]. The natural-abundance $^{13}$C NMR studies of Yehudi Levine et al. further established the mobility gradient in terms of lipid dynamics [49], where the relaxation times were found to vary moving away from the glycerol backbone toward either the polar head group or the acyl chain termini [49]. Additional insights by Melvin Klein et al. at Berkeley [50] and Ulrich Häberlen and Hans Spiess at Heidelberg [51, 52], together with pioneering work involving liquid crystals by Pier Luigi Nordio and his group at Padova [53] and by Jack Freed at Cornell [20], set the stage for the ideas brought forth in this chapter.

### 7.2.2 Order parameters and relaxation rates of phospholipid liquid crystals

Nuclear spin relaxation is notable among the various biophysical methods because both the amplitudes and rates of the motions (spectral densities) come naturally into play. That is to say, the mean-square amplitudes and correlation times at a site-directed level are involved. For membrane lipids, to establish whether the previously mentioned flexibility gradient is observed – or alternatively a mobility (fluidity) gradient – requires detailed experimental investigations. Even so, the above-mentioned $^1$H or $^{13}$C NMR relaxation studies do not readily separate the contributions from the motional amplitude (order parameter) and the motional correlation times. One breakthrough came with the implementation of solid-state $^2$H NMR spectroscopy by Seelig et al., who established detailed order parameter profiles for both phospholipids [54] and membrane liquid crystals (soap-like bilayers) [46, 55]. However, the correspondence of the flexibility gradient with the mobility gradient had to await a different type of experiment. This involved the combined solid-state $^2$H NMR order parameter and spin-lattice relaxation time measurements of Brown, Seelig, and Häberlen [56]. These authors first showed that both the order parameters and relaxation rates had gradients along the chains that paralleled one another. Besides the order profile (the flexibility gradient), there is also a relaxation profile (mobility gradient) of the lipids (Figure 7.2). The question is then whether the relaxation profile is because of the motional amplitudes, or the rates of the motions, or both [57]. That is to say: What is the functional correspondence of the two profiles, namely, the flexibility gradient and the mobility gradient?
Here we offer a new twist on the question of the mobility gradient of membrane liquid crystals: NMR relaxation studies detect the emergence of collective thermal excitations that underlie the bulk elasticity due to chain conformations over a broad time range \(10^{-11}\) to \(10^{-6}\) s. The connection of the flexibility gradient with the mobility gradient is due to collective interactions of the lipids. Order fluctuations represent elastic (harmonic) excitations of the bilayer; for example, those associated with the local chain tilt and other types of slowly relaxing or long-lived local structures (on the timescale of \(\approx 10^{-8}\) s). In consequence, the bilayer flexibility gradient depends on the timescale of the lipid deformations. Slowly relaxing conformations occur due to long-lived collective lipid interactions involving tilt of portions of the chains [37], together with other rotational isomeric configurations. Through recognizing the frequency–amplitude correspondence, one is then able to establish a connection to bulk membrane properties [58–61].

### 7.3 Flexibility and mobility of lipid bilayers as seen by magnetic resonance spectroscopy

At this point, we can briefly summarize the results of previous magnetic resonance studies (NMR and spin-label EPR) of membrane lipids as follows:

**Figure 7.2:** Experimental observables from solid-state \(^2\)H NMR spectroscopy reveal both the structure and dynamics of membrane liquid crystals. Results are summarized for binary mixtures of DMPC-\(d_{54}\)/cholesterol in the liquid-ordered (lo) phase. (a) Orientational order parameters \(S^{(i)}_{CD}\) are plotted versus acyl chain segment position \((i)\) for various mole fractions \((X_C)\) of cholesterol (Chol). Note that greater \(X_C\) yields an increase in order parameters due to loss of configurational degrees of freedom. For the initial part of the chains a plateau is seen followed by a decrease within the bilayer core. (b) Plots of corresponding spin-lattice \(R_{1Z}^{(i)}\) relaxation rates against carbon index \((i)\) for mixtures of DMPC-\(d_{54}\)/cholesterol in the lo phase. With greater \(X_C\) an opposite decrease in the relaxation rates is evident. The \(^2\)H NMR measurements were conducted for unoriented multilamellar dispersions at \(T = 44^\circ\)C and Larmor frequency \(v_0 = 76.8\) MHz (11.7 T). Solid-state \(^2\)H NMR reveals changes in both the flexibility gradient and the mobility gradient for the membrane lipids at an atomistic level. Data are from Ref Martinez et al. [60].
1. Spin-label EPR spectroscopy shows there is an increase in motional averaging of the hyperfine tensor moving toward the bilayer center, which Hubbell and McConnell [7] called the flexibility gradient. McConnell et al. [7, 8] first interpreted the flexibility gradient for phospholipids and Seelig et al. for smectic liquid crystals (soap-like bilayers) [46] in terms of orientational order parameters. The spin-label order parameters correspond to the unpaired electron of the paramagnetic oxazolidine ring ($\pi$-orbital z-axis is normal to the H–C–H plane of a polymethylene chain) [8, 55, 62, 63].

2. Analogously, proton and $^{13}$C NMR studies of lipids reveal a gradient of the spin-lattice relaxation times moving away from the glycerol backbone toward either the polar head groups or the nonpolar bilayer core, as shown by Chan et al. [47, 48, 64] and Levine et al. [49]. Still, the separation of the motional amplitudes versus the motional rates is less clear-cut than for spin-label EPR spectroscopy [8].

3. Solid-state $^2$H NMR spectroscopy as introduced by Seelig et al. determines orientational order parameters and correspondingly the motional amplitudes as in spin-label EPR spectroscopy [54, 55, 65]. However, rather than an exponential decrease along the chains [7, 46], an approximate plateau is found [54]. Coupled rotational isomerizations of the acyl groups occur [55], together with statistical chain terminations moving away from the lipid aqueous interface (end effects), as pointed out by Dill and Flory [66].

4. The mobility gradient from solid-state $^2$H NMR relaxation reveals an equivalent plateau as seen for the flexibility gradient. As first investigated by Brown et al. [56], both the order parameters and the relaxation rates show a functional correspondence, which differs from spin-label EPR results. Because the relaxation depends on both the mean-square amplitudes and the correlation times, the question is whether the relaxation gradient mirrors the order profiles (flexibility gradient) [7, 54], or whether there is a corresponding mobility gradient along the chains [56].

5. One can reduce the many-body problem of a lipid bilayer to considering the order parameters and relaxation times of the individual segments of the flexible molecules at a site-resolved level. According to this view, collective properties of the membrane lipids are significant for interpreting the results. Each of the segments is treated analogously to a nematic liquid crystal within the mean field of the bilayer, as opposed to considering the cumulative internal chain dynamics.

### 7.3.1 The flexibility gradient of a lipid bilayer

The understanding of lipid membranes clearly benefits from knowledge of both the flexibility gradient and the mobility gradient, for example, as encapsulated by molecular dynamics simulations [67–70]. To explain the flexibility gradient, one must
consider the packing of the ensemble of phospholipids, together with the chain travel (flux) away from the aqueous interface [66, 71–73]. Because of their amphiphilic character, the lipid molecules are anchored to the aqueous interface by their polar ends. The lipids are moreover subject to a balance of hard repulsive forces acting at short range that govern the average structure, together with soft van der Waals attractions at longer range, that fix the density at approximately equal to that of liquid hydrocarbon. Either a lattice model [66] or a mean-torque model [73] for the distribution of acyl chain segments is informative. The chain flux away from the aqueous interface depends on the area per molecule, which is only weakly affected by the acyl length, yet rather strongly by the polar head groups as shown by Brown et al. [73]. *Gauche* isomers shorten the individual chain length projections along the bilayer normal [55], giving a broad distribution of end-to-end lengths. Beyond a certain point (the so-called plateau), the chains with more *gauche* isomers terminate, so that the number extending deeper into the bilayer core is correspondingly reduced. The surrounding chains are more disordered to keep the density nearly the same as liquid hydrocarbon [58, 66, 71–75]. Hence the length of the order parameter plateau increases with the acyl chain length [73], and the decrease in chain order near the bilayer midplane is approximately the same for various chain-length lipids [73], as also noted by Hubbell and McConnell [7]. Packing of the chains thus accounts for the disorder (flexibility) gradient for the lipid ensemble [66, 71, 73].

Additionally, besides the much-discussed flexibility gradient, we must also consider the mobility gradient of the membrane lipids [49, 56]. Clearly the relaxation rates require further consideration, because they depend on both the motional amplitude (i.e., mean-square amplitude) and the rates of the motions [57]. One cannot a priori expect any relation between the mean-square amplitude and the rate, except perhaps for simple harmonic or anharmonic motions. Yet such a relation has indeed been discovered in the case of lipid bilayers [1]. Most surprising, the order fluctuations within the bilayer core manifest the continuum elastic properties of the bulk material (e.g., due to a distribution of harmonic excitations). Classical liquid crystal physics is uncovered, inasmuch as the results show the collective behavior of the ordered chains. For the case of phospholipids, the long acyl groups (e.g., polymethylene) are tethered to the aqueous interface. Lack of water penetration means both the configurational properties [7, 62, 66] and the dynamical properties [49, 76] of the lipids come into play, as described by the force field of the bilayer [68].

### 7.3.2 The mobility gradient of a lipid bilayer in the liquid-crystalline state

With the above-mentioned discussion in mind, the reader may ask: How is the mobility gradient of a lipid bilayer in the liquid-crystalline state connected to the flexibility gradient? In fact the author first reported the correspondence at a conference in 1979 at
Stanford University (in a session chaired by McConnell) [1]. A simple square-law dependence of the relaxation rates and order parameters was uncovered for the first time. It immediately suggested how the NMR relaxation is governed by the order fluctuations. The characteristic relation between the mean-square amplitude and the relaxation clearly points to modulating the local ordering of the bilayer lipids [1, 77]. The question then becomes: Are the order fluctuations because of non-collective molecular rotations, or to quasi-elastic (harmonic) excitations of the lipids due to their collective interactions? Indeed, the dependence of the relaxation on frequency (magnetic field strength) and the order parameters argues that collective motions account for the spin-lattice relaxation. The slow dynamics are formulated in terms of a local director axis and correspond to so-called order-director fluctuations (ODF). Of course, there is no a priori reason why this has to be so – we all know that NMR is a site-specific molecular technique [57]. So why are molecularly specific details not included?

It turns out that the concept of a director frame [71] is key to considering the bilayer relaxation due to the time-dependent lipid motions. For membrane liquid crystals, it is an axis of rotational symmetry that is perpendicular to the plane of the bilayer, that is, it is the lamellar normal [54]. Molecular motions are expressed by rotations either around (longitudinal) or perpendicular (transverse) to the principal molecular axis within the director frame. The director axis is a collective property of the lipid assembly rather than the single molecules. It implies that the ensemble-averaged motions are rotationally symmetric around a preferred axis or direction in space. Rigid body motions of the individual molecules around their long axes would be one example, but that is neither assumed nor required. Rather, the director manifests the collective rotations of the molecules and/or their flexible segments. By introducing a local director axis, one can readily account for the multi-scale dynamics of the chains within a continuum approximation, as first introduced for lipids by this author [1] (see Figure 7.1). This immediately suggests a connection between the relatively long-lived structures seen by spin-label EPR (e.g., due to rotational isomerism or local chain tilt) and the collective order fluctuations put forth to explain the spin-lattice relaxation in lipid bilayers [58]. For NMR spin-lattice relaxation, slow motions may involve transitions (timescale of $\approx 10^{-8}$ s or longer) corresponding to those detected with spin-label EPR (also with lifetimes in the range of $\approx 10^{-8}$ s) – the timescale is similar for both methods. Still, the proposal of local director fluctuations spanning a broad range of scales in space and time has remained under discussion or debate even today.

Perhaps the reader might also be inclined to ask: Did the notion of local director fluctuations emerge fully formed, just like Athena from the head of Zeus [78]? The answer is: Of course not – it was a gradual evolution, brought about by considering the various possible alternative formulations, for example, segmental versus molecular motions and so forth, beginning with the site-specific viewpoint of NMR spectroscopy. It was enforced by the fact that the spin-lattice relaxation times differ for every magnetic field strength or nucleus studied [58]. At the time, some researchers
thought it impossible to disentangle the types of motions that govern the relaxation, for example, given the debate about the spin-label flexibility gradient. How can one possibly sort out the mobility gradient measured with NMR spin-lattice relaxation?

7.4 The role of time and space in membrane biophysics

To continue further, how do we go about separating the influences of the motional mean-square amplitudes from the rates of the motions for liquid-crystalline lipid systems? Sometimes a lack of foreordained knowledge can work to one’s advantage. The conventional wisdom is that the dynamics problem for membrane lipids is intractable in analytical closed form. Yet according to a Chinese fortune cookie, one should: “Avoid unchallenging occupations – they waste your talents.” Indeed, the test is to disentangle the various motional contributions, and here the benefit of NMR spectroscopy is the ability to determine site-specific information about both order and dynamics [57].

7.4.1 Separation of dynamical and spatial variables

Now with regard to the nuclear spin relaxation, an analysis in terms of mean-square amplitudes and reduced correlation functions (or spectral densities) entails separating the coupling Hamiltonian into a time-averaged (secular) part, and a time-dependent (nonsecular) part. The average or secular part of the coupling Hamiltonian $\langle \hat{H} \rangle$ commutes with the main Zeeman Hamiltonian, and it causes the well-known energy level shifts that affect the spectral lineshape [2, 79, 80]. It corresponds to the effective Hamiltonian of McConnell and coworkers [7, 81]. The fluctuating part of the Hamiltonian $\hat{H}'(t) = \hat{H}(t) - \langle \hat{H} \rangle$ amounts to a time-dependent perturbation, brought about by the nonsecular terms that induce the nuclear spin transitions [45]. For membrane lipids, the nonsecular terms cause transitions among the energy levels, and affect the spin-lattice relaxation rate [8]. Let us first consider the average Hamiltonian: what left an early impression on this author was how both McConnell and Seelig were able to adapt the concept of an order tensor from the liquid crystal literature [82, 83] to lipid bilayers [7, 46]. For spin-labeled phospholipids [7] or soap-like bilayers (smectic liquid crystals) [46], the oxazolidine moiety is treated analogously to a nematic liquid crystal, albeit connected by covalent bonds to the rest of the chain, that is, within the force field of the membrane lipid bilayer. Then from the measured order parameter profile or flexibility gradient, one can infer the average behavior of the ensemble of lipid chains [7, 46, 84], independently of models for the cumulative dynamics.
7.4.2 Nuclear spin relaxation as a probe of lipid membrane dynamics

Continuing along these lines, provided there is a director axis, then local director excursions can also occur, which manifest the collective motions of the aligned molecules [20, 85]. Because there is an average director axis for the individual lipid segments, fluctuations of the instantaneous director can emerge from the local structure. Introducing a director frame informs the presence of collective interactions of the lipid molecules, and gives us a route to the energy landscape and the hierarchy of motions for membrane lipid bilayers. In this way, we are led to the proposal that the spin-lattice relaxation is governed by the local director fluctuations, that is, due to slowly relaxing local structures [1]. By separating the coupling Hamiltonian into a secular (effective or averaged) part and a time-dependent perturbative part, the transition probability and hence the nuclear spin relaxation rates depend on the mean-square interaction strength. The molecular fluctuations close to the resonance frequency $\omega_0$ govern the rate of the relaxation – that is, the transition probability depends on the squared matrix elements of the coupling Hamiltonian. Matching the spectral density of the fluctuations to the coherent nuclear resonance frequency $\omega_0$ (and twice $\omega_0$) causes transitions among the nuclear spin energy levels, and hence the spin-lattice relaxation. For multi-scale (or composite) motions, one can then iteratively separate the coupling Hamiltonian into various parts with characteristic timescales. What is secular for the faster timescale motion becomes nonsecular for the next slower motion in the hierarchy, and so forth [1]. Of course, cross-correlations can also be considered, but what do we gain by making a complicated problem even more complicated? Rather, our aim is simplification, leaving the details for subsequent refinement.

For the sake of illustration, let us assume a broad separation of the dynamics into faster local motions and slower motions (see Figure 7.1). The local motions (with timescales $\approx 5–20$ ps) entail rotational isomerization [86] of the lipids, which can be affected by collective interactions of the assembly due to the energy landscape [3, 56]. As an example, it is known that lateral diffusive jumps of the lipids can occur on a longer timescale ($\approx 10^{-7}$ s) [87] that can alter the rotational isomeric state or orientations of molecules. Provided the local order parameters are proportional to the observed values, the slower dynamics would affect all positions approximately equally. The secular (averaged) part of the Hamiltonian for the slow motions then scales with the local order parameter. Because the spin-lattice transition probability depends on the squared Hamiltonian matrix element, that immediately leads us to a square-law functional dependence of the relaxation profile (i.e., the mobility gradient) on the observed order parameter profile (i.e., the flexibility gradient) [58]. Notably the functional dependence of the relaxation rates on the squared order parameters along the chains [1] is completely model free. It can be further interpreted in closed mathematical form, as shown for the case of proteins in solution [88], and concurrently and more generally for aligned systems [1].
7.4.3 Non-collective or collective order fluctuations?

In hindsight, the above treatment is just a simple consequence of Fermi’s golden rule – the relaxation depends on the interaction strength (hence the coupling Hamiltonian) squared. We shall return to this point below. Given that the local isomerizations set up an ensemble of structures formulated by a local director (e.g., which might include transient local tilting of portions of the chains [58, 62, 84, 89–91]), the spin-lattice transition probability depends on the squared order parameter. It is assumed the collective director fluctuations are approximately the same within the bilayer. A posteriori the experimental observation of a simple square-law functionality of the mobility gradient on the flexibility gradient can thus be simply rationalized. Most arresting, a direct connection to lipid material properties exists, thus hinting to the physical significance of the findings. Still there is no a priori reason why this has to be so – and some would say (and did) that the problem is intractable at any level of physicochemical rigor. Why not just average the results over the whole bilayer, and calculate an average (micro)viscosity and be done with it? But that is clearly untenable as an oversimplification [58].

It is probably safe to state that many scientists would agree that the relaxation rates of lipid bilayers do not predominantly involve local segmental motions. There may be a mobility (fluidity) gradient analogous to the flexibility gradient of McConnell, but that is not the major feature [92]. In effect, the discussion revolves around the cutoff for the distance or timescales for the local director fluctuations, including whether a single type of relaxation dispersion covers the whole frequency range. The cutoff for the ODF can be on the order of the bilayer thickness, in which case the dispersion at higher frequencies can be due to whole-molecule motions. Alternatively, the various segments can be considered analogously to a nematic liquid crystal, subject to an orienting potential with a cutoff at the segmental size, yielding the emergence of collective thermal fluctuations of the local director. A break in dimensionality can occur from 3D order fluctuations to 2D order fluctuations of lower amplitude, for example, connected with undulations of the membrane lipid bilayer at low (kHz) frequencies. (For now, we put aside whether the small-angle approximation for the ODF is applicable [93].) Assuming the bilayer dynamics involve both local motions (e.g., trans-gauche isomerizations) as well as order fluctuations, the issue boils down to whether the slow dynamics can be formulated by a non-collective model, for example, rigid-body molecular rotations in a potential of mean force, or whether a broad distribution of quasi-elastic excitations is present. Indeed, the author extensively discussed both alternatives [1], and it was argued that collective lipid dynamics are the most plausible interpretation.

Basically the reasoning goes as follows: effectively, there is a continuous frequency dispersion of the relaxation, yet the temperature variation by contrast implies the fast motional (white noise) limit. How can such apparently contradictory observations be reconciled, for example, by a model of rotational diffusion? Then again, evidence for distinct rotational modes in lipid bilayers (as shown by a minimum in
the spin-lattice relaxation times) has never been obtained for phospholipids in the liquid-crystalline, that is, liquid-disordered (ld) state. (Such a relaxation minimum is observed due to matching of the spectral density of the rotational modes to the nuclear resonance frequency.) It is only for the phosphodiester moiety that such a minimum is seen [94], or for bilayers containing cholesterol [95, 96]. Even in the case of inertial averaging of the lipids over the internal (fast) degrees of freedom, whole-molecule rotations of the lipids are not detected in the liquid-crystalline state. Apparently a reduction in the degrees of freedom is needed for rotational modes to be observed. How could such discrete whole molecule motions exist, and not give rise to a relaxation minimum at some combination of frequency and temperature?

7.5 The energy landscape of a membrane lipid bilayer

Outwardly the more interesting (in our view) alternative is a hierarchical energy landscape for the dynamics of lipid membranes. The various tiers correspond to the fast and slow motional modes [1, 60, 97, 98] that occur within the basins of attraction. Here, we are much inspired by work involving protein dynamics by Hans Frauenfelder and coworkers [99–101]. To all appearances, the hierarchy shown in Figure 7.1 can be viewed as a low-dimensional representation of the energy landscape for the flexible membrane lipids in the liquid-crystalline (i.e., ld) state. The individual C–H bonds can fluctuate due to vibrational motions, trans-gauche isomerizations, and restricted segmental reorientations (ps time range). Fluctuations in local ordering of the lipids can also occur due to molecular motions (e.g., involving inertial averaging, ns range), or collective lipid dynamics over a broad span of timescales (ns–μs or ms range, and beyond). Structural and dynamic features are explored using various biophysical techniques, including small-angle X-ray and neutron scattering (SAXS, SANS), molecular dynamics (MD) simulations, and magnetic resonance (spin-label EPR and NMR) spectroscopy.

7.5.1 Hierarchical models for lipid membrane dynamics

This thinking leads us to the idea of a separation of relatively fast (local) and slow (collective) fluctuations, that in a first approximation are uncoupled (statistically independent) [10]. What are the classes of models that can be considered? Broadly speaking, the fast motions set up the local ordering that is further modulated by slower collective disturbances. Yet as mentioned above, for membrane lipids, the slower order fluctuations have been the topic of intense debate. Some authors [1] claim that a distribution of collective excitations occurs in analogy with nematic and smectic liquid crystals. The relatively slow motions are formulated as order-director
fluctuations, spanning the segmental dimensions on up to the macroscopic bilayer. Still other authors consider inertial averaging over the entire lipid molecule, that is, a non-collective molecular model is assumed, whereby the average molecule undergoes rotational diffusion within the mean field of all the other lipids of the bilayer. For the higher frequency spin-lattice relaxation (MHz) regime, many workers in the field accept an interpretation in terms of rotational modes of the lipids [102, 103]. As first proposed by the author for lipid bilayers [1], the possibility of ODF is mainly considered for the lower frequency (kHz) range, where the order fluctuations are relatively small in amplitude [104].

Coming back to Figure 7.1, such motions affect the coupling interactions in NMR spectroscopy, which correspond to a static or time-dependent perturbation of the Zeeman Hamiltonian. The coupling interactions are formulated as second-rank tensors, and are represented in either a Cartesian or spherical (irreducible) basis [2, 80]. By introducing the closure property of the group of rotations [105], consideration of the multi-scale motions then becomes transparent, simplifying the overall treatment of the problem [80, 106]. Besides the local segmental motions of the lipids, molecular motions can occur with respect to a local director axis \( n(t) \) that itself can undergo time-dependent reorientation (Figure 7.1). For membrane lipids, the residual quadrupolar couplings (RQCs) or residual dipolar couplings (RDCs) directly give the order parameters for flexible molecules as model-free experimental observables. The nuclear spin relaxation rates depend on the types of the lipid or protein motions, as well as their rates and mean-square amplitudes (related to the order parameters) [1, 21, 23, 24, 27, 57, 88, 107, 108]. Both equilibrium structural properties and dynamical properties are thereby accessible [57]. For liquid-crystalline membranes, the motions encompass local isomerizations of the flexible lipids, rotations of the entire molecules, and collective excitations of the whole bilayer, for example, involving a distribution of quasi-elastic relaxation modes, as in other liquid crystals. Collective director fluctuations occur with respect to the average director \( n_0 \) (the bilayer normal), and are due to the distribution of either slowly relaxing local structures (ODF), or non-collective molecular motions [1].

In the case of phospholipid membranes, samples are typically aligned bilayers, multilamellar dispersions, or unilamellar vesicles. Lipid bilayers are ordered systems with a large number of internal degrees of freedom, and they typically reorient slowly on the NMR timescale. What is more, in solid-state NMR spectroscopy the symmetry axis (bilayer normal) can be aligned with respect to the magnetic field. Because the motions are restricted, the transformation between the principal axis system of the coupling interaction (dipolar, quadrupolar, and chemical shift) and the bilayer frame is only partially averaged, for example, by segmental isomerizations or molecular rotations. Larger scale motions involve collective disturbances of the bilayer. Assuming statistically independent fluctuations (e.g., with separate timescales), a hierarchical two-step relaxation model can be introduced. Such a two-step separation of the relaxation is implicit in considering the multi-scale motion [109]. First,
relatively fast motions of lesser amplitude modulate the static coupling interaction about the average value. And second, slower motions of greater amplitude further modulate the average or residual interaction over the faster motions, and furnish a second relaxation contribution due to the order fluctuations. In the limit of isotopic or unrestricted motion, a generalized model-free (GMF) approach [27] gives a two-step model, as put forward by Håkan Wennerström et al. for surfactant micelles [110], or the model-free approach of Giovanni Lipari and Attila Szabo for globular proteins in solution [88, 111].

Experimentally, the order parameters and relaxation rates describe the coupling interactions – they are the observables of the solid-state $^2$H NMR experiments. The GMF reduction of the $^2$H and $^{13}$C NMR lineshape and relaxation measurements involves the motional mean-square amplitudes (order parameters) and reduced spectral densities (correlation times). Direct dipolar couplings and quadrupolar interactions are measured, together with the nuclear spin relaxation rates. The segmental order parameters give information about the average bilayer structural properties, including the area per lipid and volumetric hydrocarbon thickness, as evaluated using a mean-torque model [73]. Fluctuations about the mean value, for example, area or thickness fluctuations, or director fluctuations, yield the multiscale bilayer dynamics. What is more, by combining the RQCs or RDCs with the associated spectral densities, a unified picture of the protein and lipid dynamics can be developed. The NMR relaxation rates give us knowledge of the types, rates, and amplitudes of the collective or molecular motions within the bilayer. Measuring both the experimental $^{13}$C–$^1$H and $^2$H NMR segmental order parameters ($S_{CH}$ or $S_{CD}$) and relaxation times ($T_{1Z}$, $T_{1Q}$, $T_2$) allows findings to be obtained for membranes that cannot be acquired with other biophysical techniques. In this context, a comprehensive database of experimental NMR results has been developed for membrane phospholipids [32].

### 7.5.2 Example of bilayers containing cholesterol

To illustrate these ideas further, it is instructive to consider the interactions of phospholipids with cholesterol [61, 70, 112–117], a molecule in which McConnell had a longstanding interest [14, 16, 118–120]. In solid-state NMR, the motional averaging gives a distribution of RQCs, from which the order profiles can be derived (Figure 7.2). The static electric field gradient tensor is essentially the same for all the deuterated positions [2]. Yet the residual tensor is different: it varies strikingly as a function of acyl chain position, giving the RQCs that naturally draw our attention. A further aspect is that the solid-state $^3$H NMR spectra clearly exhibit the axial symmetric signature of the liquid-crystalline (Id) phase of lipid bilayers. As famously shown by Myer Bloom and his coworkers [121], for acyl chain perdeuterated phospholipids, the powder-type spectra of random multilamellar dispersions can be
numerically inverted (or de-Paked) to reveal the RQCs most directly [122]. The larger splittings correspond to the groups closer to the aqueous interface, with a progressive diminution along the chains, toward the hydrocarbon core of the bilayer [7, 8, 46]. From the de-Paked $^2$H NMR spectra, order parameter profiles $S_{CD}^{(i)}$ (where $i \equiv$ chain index) are obtained for the case of random multilamellar dispersions [2].

Some readers will know already that the residual couplings (RQCs and RDCs) are simply related to the average bilayer structure, for example, in terms of a mean-torque potential model [73]. Insights are obtained about the structure and dynamics of the membrane lipids in terms of the mean-square amplitudes and rates of the motions. Returning back to Figure 7.2a, a progressive increase in the order parameters is seen with a greater mole fraction ($X_C$) of cholesterol. Interaction with the rigid sterol frame yields a pronounced reduction of the degrees of freedom of the flexible phospholipids. The effect of cholesterol is to increase the order parameters proportionately for the various acyl segment positions. The well-known condensing effect (decrease in area per molecule) is due to an increase in the acyl chain projections along the bilayer normal (director) [73]. By contrast, for the polar head group segments there is no such increase in the order parameters, as first shown by Brown and Seelig [112]. Because of the umbrella effect, the rigid sterol moiety is situated deeper in the bilayer – it acts as a spacer insofar as the polar head groups are concerned [112].

Analogous measurements of the nuclear spin relaxation rates are possible by perturbing the spin system with a suitable radiofrequency pulse sequence, and then following the return back to equilibrium [60]. Both the $S_{CD}^{(i)}$ order parameters and $R_{1Z}^{(i)}$ relaxation rates for multilamellar lipid dispersions decrease along the acyl chains as a function of segmental index ($i$) in the liquid-crystalline (ld) state, that is, on going from the aqueous interfacial region toward the bilayer hydrocarbon core (Figure 7.2). The approximate plateau for both experimental observables as a function of segmental position is because of the effects of tethering the acyl chains to the aqueous interface [55, 56]. The order profiles for DMPC-$d_{54}$ show an increased disorder toward the bilayer center, accompanied by a reduction in the acyl segmental relaxation rates. Most arresting, as the molar ratio of cholesterol in the bilayer increases, there is an opposite effect on the NMR observables: in Figure 7.2a the order parameters increase, while in Figure 7.2b there is a reduction in the spin-lattice relaxation rates. How can we explain these apparently contradictory experimental results?

7.5.3 Space and time revisited

Let us next return to the question: What are the types of motions that govern the properties of phospholipid membranes, including their rates, amplitudes, and energetics? And how can we explain the opposite influences of cholesterol on the acyl segmental order profiles compared to the relaxation rate profiles? In discussing the lipid conformations in liquid-crystalline phases, the various local structures can be
expected to have different lifetimes. It is then paramount to consider the spectroscopic timescale used to investigate the distribution. Undoubtedly, in the case of NMR and spin-label EPR, the couplings involve direct and indirect dipolar (hyperfine) interactions, quadrupolar interactions, and chemical shielding (Zeeman) interactions. For comparing spin-label EPR studies to NMR studies of lipid bilayers, the magnitudes of the various couplings need to be kept in mind. The reason is that structural transitions can occur with rates greater than or approximately equal to the anisotropy of the coupling (e.g., dipolar, quadrupolar, and chemical shift), leading to averaging of the interactions. McConnell pointed out this aspect very clearly when he stated:

The angular brackets... denote a time average over a characteristic time T. For nitroxide spin labels the time T is determined by the reciprocal of the anisotropy of the hyperfine interaction and Zeeman interactions, in frequency units. This places T in the range $10^{-7}$–$10^{-9}$ sec [8].

In the example of spin label EPR, the reciprocal anisotropy of the hyperfine coupling is approximately $1/(T_{\parallel} - T_{\perp})$ and falls in the range of $\approx 10^{-8}$ s [123]. This timescale is comparable to the motions detected by NMR spin-lattice (Zeeman) relaxation, which also detects motions in the range of $1/\nu_0 = 10^{-8}$ s [1]. Even so, the anisotropy of the static quadrupolar coupling in solid-state $^2$H NMR spectroscopy is smaller, and is $1/[\nu_0^2]_{\parallel} - [\nu_0^2]_{\perp} \approx 5 \times 10^{-6}$ s for the two individual spectral branches [80].

We can then ask: How are the NMR results connected to those of spin-label EPR spectroscopy? Certainly, it is thought provoking that spin-label EPR and $^2$H NMR spin-lattice relaxation both detect motions on a similar timescale, while solid-state $^2$H NMR spectra include additional motions at lower frequencies. Here the introduction of a local director can aid in further explaining the spectroscopic observables. Spin-lattice ($T_{1Z}$) measurements detect fluctuations near $1/\nu_0 = 10^{-8}$ s as already mentioned earlier, which is near the limit of the motional averaging in conventional EPR spectroscopy [8]. Provided the relaxation entails ODF, conformations with lifetimes of $\approx 10^{-7}$–$10^{-9}$ s that are long-lived for spin-label EPR spectroscopy might contribute to the nuclear spin-lattice relaxation, yet be further averaged by the more than 100-fold greater timescale in solid-state $^2$H NMR spectroscopy. Local perturbations in the vicinity of the spin label may also be involved [54, 124]. Here we simply note that for lipid bilayers the concept of a local director axis [1] implies it can vary in both space and time. One should also recall that the director axis is a property of the phase, rather than an individual molecule (see above discussion). It depends on the assembly of flexible molecules within the bilayer, for example, the local director can vary over small distances, approaching the segmental or molecular size [58]. The presence of a local director frame (e.g., due to slowly relaxing local structures of the lipids) might explain why transient local chain tilting can occur in the vicinity of a spin label, which might not be detected by solid-state $^2$H NMR because of the longer timescale [1].
7.5.4 Dimensionality of the order-director fluctuations

Moreover, the dimensionality of the order fluctuations can depend on the spectroscopic timescale involved. The next question is whether a 3D membrane deformation model or a 2D flexible surface model is appropriate. As an example, the spin-lattice ($T_1Z$, Zeeman) measurements are sensitive to the spectral density of the motions near $\approx 10^{-8}$ s, and can detect 3D ODF (quasi-nematic) at the level of the local structures of the hydrocarbon chains (e.g., as seen by spin-label EPR). For 3D ODF the elastic fluctuations might not be so strongly constrained by the polar interface with water, in contrast to 2D fluctuations due to the membrane surface. One can also consider that there are high-frequency ODF [1] but their dimensionality is 2D, as for a smectic liquid crystal, rather than 3D as for a nematic liquid crystal. Alternatively there may be no high-frequency ODF at all – rather, a non-collective model describes the order fluctuations in the MHz range, due to whole-molecule rotational modes of flexible lipids.

Still, a bilayer has a finite thickness as investigated experimentally by X-ray and neutron scattering methods [73, 125]. Collective motions over larger distances can then evolve from 3D into 2D ODF (smectic-like) that are governed by the bending rigidity, as in the case of lower frequency transverse relaxation ($R_2$) measurements (kHz range). Starting at high frequencies, an $\omega^{-1/2}$ law can apply (MHz range) due to quasi-nematic ODF at short distances ($\lambda < t$), as first discussed by the author [1], where the local director might not extend beyond the bilayer thickness ($t$). As the nuclear resonance frequency (magnetic field strength) decreases over longer distances on the order of the bilayer thickness and beyond ($\lambda > t$), the dynamic behavior can increasingly pick up smectic-like character, due to the boundary condition of the aqueous interface. The fluctuations can then involve smectic-like ODF at lower frequencies, where an $\omega^{-1}$ law occurs (kHz range) due to bending of the membrane interface with water [104, 126]. The idea of collective disturbances of the bilayer has been subsequently adopted in work involving transverse nuclear spin relaxation [127–130]. It is assumed that the elastic modes occur at long wavelengths and low frequency (kHz), because the molecular motions yield a cutoff for order fluctuations in the MHz range. The low-frequency motions amount to 2D undulations of a flexible surface of relatively small amplitude, as opposed to 3D ODF of potentially larger amplitude. Such collective motions can be important for processes involving membrane fusion, interbilayer forces, and lipid–protein interactions.

At this stage we conclude that the introduction of a local director can account for slowly relaxing local structures (e.g., transient local tilt of portions of the chains) [1, 58, 131]. The idea of a broad distribution of collective bilayer tilt disturbances suggests there may be two regimes of the relaxation dispersion due to harmonic or elastic excitations. Depending on the cutoff length, either nematic-like or smectic-like fluctuations can occur due to the hydration levels and the types of polar head groups. In the high frequency MHz regime, primarily 3D collective fluctuations of the local structures govern the spin-lattice relaxation, which overlaps the spin-label EPR
timescale [32, 132]. Contributions from collectively tilted local structures, with life-
times intermediate between the $^2$H NMR ($\approx 10^{-5}$ s) and spin label EPR ($\approx 10^{-7}$–$10^{-9}$ s) timescales, might average the $^2$H NMR quadrupolar coupling, but not the $^{14}$N hyper-
fine interactions in spin-label EPR spectroscopy [62, 84]. At longer distances, the bilayer aqueous interface can yield a transition from a frequency dispersion governed by 3D ODF to 2D ODF as the limit. In the lower frequency kHz range, a 2D collective model may then be applicable corresponding to transverse NMR relaxation time studies [104, 128, 130].

### 7.6 The paradigm shift to order-director fluctuations

The preceding discussion builds on the idea of a broad distribution of harmonic or
quasi-harmonic (elastic) excitations in lipid bilayers, as originally put forth by the
author [1]. As an approximation, we can neglect both the short and long wavelength
cutoffs for the distribution, which are taken as zero and infinity, respectively. It is
also possible that breaks in the frequency dispersion law (power spectral density)
can occur. For example, there can be a transition from the regime of 3D ODF
(membrane deformation model) with an $\omega^{-1/2}$ dispersion law to the 2D ODF regime
(flexible surface model) having an $\omega^{-1}$ power law [126, 132]. In effect, the long
wavelength cutoff for the 3D regime becomes the short wavelength cutoff for the
2D fluctuations (e.g., on the order of the bilayer thickness). Moreover, the elastic
constants and amplitudes of the fluctuations can be different. According to this
view, the 3D regime has an $\omega^{-1/2}$ dependence in the MHz range that overlaps the
spin-label EPR timescale. In the lower frequency kHz range, the properties of the
aqueous interface give an increasingly smectic-like behavior, with an $\omega^{-1}$ disper-
sion law [126, 132]. The 2D collective motions include flexible surface undulations
that contribute mainly to the transverse ($T_2$) relaxation in NMR, for example, they
can show up as inhomogeneous line broadening [8] in spin-label EPR
measurements.

The idea of a local director axis that extends over a portion of the lipid or the bilayer
thickness can be helpful to understanding the multi-scale physics of the bilayer fluctua-
tions involving slowly relaxing local structures. For a 3D collective model (i.e., membrane
deformation model), the relaxation dispersion scales as $\omega^{-1/2}$ [1, 58, 132, 133], whereas
for 2D collective motions (i.e., flexible surface model), the relaxation goes as $\omega^{-1}$ [104,
126, 130, 132]. One can then ask: What is the contribution to the relaxation rates from
ODF assuming plausible values for the various physical constants? For a 3D collective
model, the properties of the hydrocarbon core are considered. The theoretical viscoe-
elastic coefficient assuming an $\omega^{-1/2}$ frequency (magnetic field) dispersion law is
$D = (3/5)k_B T/\pi S_{\text{slow}}^2 (\eta/2K^3)^{1/2}$, while the experimental value for an $\omega^{-1/2}$ power-law
dispersion is $D = 2 \times 10^{-5}$ s$^{1/2}$ [132]. Substituting $S_{\text{slow}} \approx 0.6$ for the slow order
parameter [1, 132], a viscosity of \( \eta \approx 1 \text{ cP} \), and an elastic constant of \( K \approx 0.2 \times 10^{-11} \text{ N} \) gives a value of \( D = 1.7 \times 10^{-5} \text{ s}^{1/2} \) in agreement with 3D collective motions. (No distinction is made between twist, splay, and bend modes, and moreover the compression modulus \( B \) may come into play [41].) It follows that the physical significance of a 3D collective ODF model is supported by its consistency with the relevant material constants.

On the other hand for a 2D collective model (i.e., flexible surface model), properties of the bilayer interface with water may become an important factor. Fluctuations of a deformable surface are involved (despite that the bilayers have a finite thickness) [73, 125]. The theoretical value of the elastic coefficient (there is no viscosity due to lack of coupling to a third dimension) is \( D' = \left( \frac{3}{5} \right) \left( \frac{k_B T}{S_{\text{slow}}} \right)^2 / 2K_C \), while the experimental value assuming an \( \omega^{-1} \) power-law dispersion is \( D' \approx 0.5 \) [132]. Inserting experimental values or estimates of \( S_{\text{slow}} \approx 0.6 \) [132] and a curvature elastic modulus (bending rigidity) of \( K_C = 0.6 \times 10^{-19} \text{ J} \) [135] yields \( D' = 0.06 \), which is about an order of magnitude smaller than the experimental value. Apparently an \( \omega^{-1} \) power-law dispersion in terms of undulations does not fit the experimental frequency (magnetic field) dependence of the spin-lattice relaxation in the high-frequency MHz regime [132, 136]. Then again, it may be appropriate for lower frequency (kHz range) motions, as studied by transverse Carr–Purcell–Meiboom–Gill (CPMG) relaxation dispersion experiments [104, 128, 137].

As a matter of fact, the idea of collective motions as governing the relaxation has been surprisingly controversial. Although the formulation of ODF for lipid bilayers as first proposed for high-frequency motions [1] has been debated, the identical concept has been reintroduced subsequently when it comes to lower frequency motions. In keeping with this view, the high frequency dispersion (MHz range) includes non-collective molecular rotations within the force field of the membrane lipid bilayer. Collective membrane motions contribute to the relaxation, but only at low frequencies (kHz range), where their dimensionality is 2D and they are of small amplitude. Evidently, the debate centers around the amplitude and timescales of the collective disturbances, and their connection to magnetic resonance observables (solid-state NMR or spin-label EPR spectroscopy). A further aspect is that a simple physical significance of the theoretical approach should emerge in combination with experimental measurements [58].

### 7.6.1 Fermi’s golden rule

Consistent with the above-mentioned thinking, NMR spectroscopy provides us with essential experimental information about the structural dynamics of biomolecules that is largely unobtainable with other biophysical methods. For flexible membrane lipids, a distribution of RQCs or RDCs is evident that manifests the bilayer structural quantities, such as the area per lipid at the aqueous interface and the volumetric...
bilayer thickness [73]. Most significantly, the NMR relaxation times tell us about the
types of motions that yield the motional averaging within the membrane bilayer,
including their amplitudes and rates. The question is then whether the NMR relaxa-
tion reports on the local segmental lipid motions, or rather the collective or molecular
fluctuations of the lipids. Put in the language of NMR spectroscopy: Does the
relaxation entail modulation of the static coupling, or the residual couplings pre-
averaged by faster segmental motions? Moreover: What is the connection to bilayer
material properties and/or biological functions?

It is here that the influences of bilayer additives such as cholesterol [138] or
nonionic surfactants [139] can further inform the analysis of bilayer fluctuations, as
seen by magnetic resonance spectroscopy. Undeniably, cholesterol is one of the
most important chemical compounds found in association with the lipid hydro-
carbon chains in a biological setting [61, 138, 140, 141]. Very few biomolecules have
been scrutinized to the same extent as cholesterol, for example, as in the studies
conducted by McConnell et al. [14, 142]. The sterol intercalates between the lipids,
and causes a dramatic stiffening of the bilayer in conjunction with the well-known
condensing effect. Coming back to Figure 7.2 as a specific example, a clear influence
of cholesterol is evident for both the order profiles and the relaxation profiles. Most
conspicuous, there are opposite effects of cholesterol on the order parameters ($S_{CD}$)
and spin-lattice ($R_{1Z}$) relaxation rates, as already noted. For cholesterol-stiffened
bilayers, large absolute order parameters and low relaxation rates are measured.
But as the mole fraction of cholesterol $X_C$ in the lipid mixtures increases, the
$R_{1Z}$ rates become smaller – exactly opposite to the effect of cholesterol on the
order profiles in Figure 7.2a. How can we explain these apparently contradictory
findings?

Indeed, Fermi’s golden rule gives a simple explanation of the correspondence of
the relaxation rates and the order parameters, whereby the spectral transition prob-
abilities depend on the squared interaction strength. Referring now to Figure 7.3, we
see that theory predicts – and experiments confirm – a remarkably simple square-law
functional dependence for the relaxation due to slow order fluctuations. For if we
transform the same data in Figure 7.2 into a square-law plot, the previously confusing
results fall neatly into line with the stiffening effect of cholesterol on the bending
rigidity of the membrane [135]. The bilayer lipids modulate the residual couplings left
over from the local segmental isomerizations, for example, ODF, as first proposed by
the author [1]. Most arresting, the experimental results in Figure 7.3 are totally model
free. We can conclude from the square-law signature [32, 77] that the relatively slow
order fluctuations govern the NMR relaxation of lipid bilayers [1, 132]. Local segmental
motions modulate the same static coupling tensor for all the segment positions,
while the slow order fluctuations involve the residual couplings pre-averaged by the
faster local motions. Modulation of the site-specific couplings remaining from the
local segmental motions is what produces the NMR relaxation for the membrane
lipids – what we call order fluctuations [1].
7.6.2 Quasi-elastic bilayer deformation

What is the physical significance of the model-free square-law functional dependence? To continue, Figure 7.3 illustrates how the square-law slopes observed for DMPC-\(d_{54}\) bilayers depend on the presence of either cholesterol or nonionic surfactants. One indication is a progressive diminution in the square-law slopes for the bilayer liquid-ordered (lo) phase with increasing mole fraction of the rigid cholesterol molecules, matching its effects on the bulk bilayer elasticity (Figure 7.3). Lanosterol is known to stiffen the lipid bilayer less than cholesterol [143, 144], which also agrees with the solid-state \(^2\)H NMR analysis [145]. A plausible inference involves the elastic properties of bilayers containing lanosterol versus cholesterol [146, 147]. Then again, an opposite softening is produced by nonionic surfactants such as C\(_{12}\)E\(_8\), which are chemical compounds important for pharmaceutical and cosmetic formulations [139, 148]. Here also a clear parallel exists with the NMR square-law slope for the combined order parameter and relaxation observables (Figure 7.3) [98, 145, 149]. Low absolute order parameters are observed for surfactant-softened bilayers, along with enhanced relaxation rates, giving a large square-law slope (Figure 7.3). The increase

Figure 7.3: Functional dependence of solid-state \(^2\)H NMR relaxation rates and order parameters reveals collective fluctuations in lipid membranes. Experimental data are the same as in Figure 7.2. The \(^2\)H \(R^{(i)}_{1\Z}\) relaxation rates are plotted versus the corresponding \(|S^{(i)}_{CD}|^2\) values for acyl segments of DMPC-\(d_{54}\) with different molar ratios of cholesterol or detergent. The spin-lattice relaxation rates \(R^{(i)}_{1\Z}\) (mobility gradient) depend on the squared order parameters \(S^{(i)}_{CD}\) (flexibility gradient) for the individual acyl segments (index \(i\)). Changes in square-law slope correspond to the macroscopic bilayer elasticity. Lipid membranes are stiffened by cholesterol (Chol) and yield a progressive reduction in slope in the lo phase. By contrast, softening by the nonionic surfactant C\(_{12}\)E\(_8\) gives an opposite increase in square-law slope. The \(^2\)H NMR measurements were conducted at \(T = 42\) or \(44\) °C and 76.8 MHz (11.7 T). Data are replotted from Refs Martinez et al. [60] and Otten et al. [149].
is due to the greater number of degrees of freedom arising from the chain configurational disorder. Opposite influences of detergents and sterols underlie detergent-resistant, raft-like lipid microdomains in biomembranes [150]. (Here we recall that like dissolves like as in our introductory chemistry courses.)

From the previously mentioned results, it follows that the model-free, square-law functional dependence of the relaxation ($R_{12}$) rates and the order parameters ($S_{CD}$) can be interpreted by quasi-elastic deformations of the lipid bilayer. Such harmonic excitations occur on the mesoscopic length scale of the bilayer thickness, and even less [1]. The combined order parameters ($S_{CD}$) and relaxation rates ($R_{12}$) inform the stiffening or softening of the membrane that emerges from the local atomistic level interactions. Changes in membrane properties are explained using a theoretically predicted, square-law proportionality between the RQCs and relaxation rates, as first proposed [1]. The combined $^2$H NMR relaxation and order parameter data manifest the order fluctuations due to the collective interactions of the entangled membrane lipids. Even more striking is the correspondence of the atomistic NMR observables with bulk material properties, for example, as investigated using micropipette deformation [135], or shape fluctuations of giant unilamellar vesicles [151, 152]. The opposite effects of cholesterol and detergents — while maintaining a square-law dependence — are an arresting discovery. It supports our original contention that the NMR observables manifest quasi-elastic bilayer deformation on the mesoscopic and even atomistic level [1]. Hydration-mediated collective dynamics of phospholipid membranes are similarly revealed by $^2$H NMR transverse relaxation rate ($R_2$) measurements [153]. Essentially the same intermolecular forces and potentials govern the atomistic-level fluctuations, as well as deformation of the membranes by an external disturbance (fluctuation–dissipation theorem).

### 7.6.3 Relaxation dispersion and unification of experimental relaxation laws

Up until now we have tended to focus on the square-law signature of relatively slow order fluctuations of membranous soft matter. Still, in membrane biophysics the dependencies of the relaxation rates on frequency (magnetic field strength) and ordering (mean-square amplitude) are likewise pivotal to interpreting the spectroscopic observables. We next put forth a unified view of the combined frequency (magnetic field) and order dependence of the relaxation. Clearly, the dynamics of membranes are the same when studied using either $^2$H or $^{13}$C spin probes. Yet the power spectral densities of the fluctuations for the two nuclei near the resonance (Larmor) frequency vary on account of the different coupling mechanisms. The frequency dispersive behavior of the relaxation depends on the nucleus studied. In consequence, by analyzing the relaxation data for the two nuclei, we can cover a larger effective frequency interval than for a single nucleus alone. Fits of the $^2$H and $^{13}$C relaxation rates to a
composite membrane model yield the contributions of fast local segmental motions ($R_{1Z}^{\text{fast}}$) and slower motions ($R_{1Z}^{\text{slow}}$) to the frequency dispersion. Cross-correlations are neglected as a first approximation, giving $R_{1Z} = R_{1Z}^{\text{fast}} + R_{1Z}^{\text{slow}}$ for the observed relaxation rate [1, 106]. The order parameter $S_{\text{fast}}$ describes local segmental motions, and the $S_{\text{slow}}$ order parameter includes the slower motions (either molecular or collective). Here $S_A = S_{\text{fast}}S_{\text{slow}} = \langle P_2(\cos \beta_{PD}) \rangle$ where $S_A \equiv S_{\text{CD}}$ or $S_{\text{CH}}$ and the rotational correlation time $\tau_C$ is on the order of $\approx 10^3$ bond vibrational periods. The local dynamics do not appreciably affect the frequency dispersion in the short correlation time limit. However, slower motions such as non-collective molecular rotations and collective bilayer excitations contribute to the frequency dispersion of the $R_{1Z}^{\text{slow}}$ relaxation rate.

Additionally, the range of the frequency dispersion can be expanded through consideration of the multinuclear spin relaxation rates. The overall relaxation rates arising because of molecular motions $R_{1Z}^{\text{mol}}$ and/or collective motions $R_{1Z}^{\text{col}}$ can be investigated in terms of a unified $^{13}$C and $^2$H frequency scaling law [32, 154] (see Figure 7.4). The scaled rates (denoted here by $\tilde{R}_{1Z}$) show the unification of the quadrupolar and dipolar relaxation for the acyl group (CH$_2$)$_n$ resonance of the DMPC bilayer in the $\alpha$ phase. It is arresting that the $^2$H and $^{13}$C spin-lattice relaxation rate dispersions measured for the same lipid segments collapse to a single plot as shown in Figure 7.4a. A scaled or reduced relaxation rate $\tilde{R}_{1Z}$ is obtained, such that a

![Figure 7.4](image)

**Figure 7.4:** Multinuclear spin relaxation rates for liquid-crystalline bilayers are unified by a simple frequency power-law. Scaled $^2$H NMR and $^{13}$C NMR spin-lattice relaxation rates $\tilde{R}_{1Z}$ are compared ($v_0 \equiv v_C, v_D$) and collapse to a single curve. (a) Relaxation rate dispersions for natural abundance DMPC and isotopically enriched 1, 2[$^3$'-$^3$]-$^2$H] DMPC are shown at $T = 30$ °C. The power-law dispersions for the C3 position in $^{13}$C NMR and $^2$H NMR are fit by a single power-law function (---) with $n = -\frac{1}{2}$ as the exponent for 3D collective order fluctuations. (b) Double-logarithmic plots of scaled relaxation rates fit to various power-law frequency scalings are shown. Power-law exponents are shown for $n = -2, -1, -\frac{1}{2}$ due to non-collective molecular rotations, 2D collective order fluctuations (flexible surface model), and collective 3D order fluctuations (membrane deformation model). A 3D collective model describes the frequency (magnetic field) dispersion of the relaxation. Figure adapted from Ref Leftin and Brown [32].
simple power-law trend is followed for the collective dynamics, as shown in
Figure 7.4b. Notably the combined dispersions obey a three-dimensional power-law
\((\omega^{-1/2}; d = 3)\) spanning nearly the full MHz regime, from \(\omega_0/2\pi = 2\) MHz to \((\omega_c + \omega_H)/2\pi = 939\) MHz for the carbon acyl chain segments [132]. Evidently it follows that a
simple 3D collective model (membrane deformation model) can unify the combined
\(^2\)H and \(^{13}\)C spin-lattice \((R_{1Z})\) relaxation rates, consistent with quasi-elastic excitations
as the origin of the relaxation in the liquid-crystalline state. Although an \(\omega^{-1}\) fre-
quency dispersion has also been proposed [96, 126, 130], it does not seem to be
supported for the acyl chain segments. We can conclude at this point that the
measurable relaxation rates are described by a simple relaxation law of the form
\[ R_{1Z}^{(i)} = A\tau^{(i)} + B|S_{CD}^{(i)}|^2\omega^{-1/2} \] where \(A, B\) are constants, which encapsulates the depend-
ence on the order parameter and the resonance frequency [1].

Nevertheless with regard to the phospholipid head groups, a different experimental
relaxation law has been observed for lipid bilayers in the case of transverse relaxation
time measurements. A 2D power-law scaling \((\omega^{-1}; d = 2)\) for the \(^{31}\)P NMR phospholipid
head group dynamics has been identified by transverse relaxation \((T_2)\) measurements in
the kHz regime by Gerd Kothe and his coworkers [130, 137], while for the acyl chains such
a relaxation law is not supported by the experimental data as shown in Figure 7.4b.
Interestingly, the difference in power-law scaling may reflect the greater sensitivity of the
head group \(^{31}\)P nucleus to the surface or smectic-like undulations. By contrast, measure-
ments using \(^2\)H or \(^{13}\)C NMR as a probe of the acyl chain dynamics may be indicative of the
3D interior of the bilayer hydrocarbon core. Despite the changed power-law exponent,
the analysis suggests that the nonexponential relaxation dispersion arises from low-
frequency motions due to highly damped ODF, as first proposed [1]. An alternative
involves distinct rotational modes of the head group segments [94, 102], as discussed
subsequently.

### 7.7 Collective or non-collective lipid motions revisited

In the case of phospholipids the acyl chains are highly entangled, and additionally the
polar head groups have dipolar and hydrogen-bonding interactions with the neighbor-
ing molecules. What are the types of molecular motions that occur in fluid, liquid-
crystalline bilayers? Let us now return to the question of whether we can identify
discrete modes for the rotational dynamics of phospholipids in the liquid-crystalline
state. The most arresting distinction is whether the dynamics correspond to a non-
collective molecular model, for example, as in the case of molecular rotations within
the potential of mean force of the lipid bilayer. Here the moments of inertia are
averaged over the internal degrees of freedom of the molecule. Alternatively, as
described above, we can consider the quasi-elastic (harmonic) excitations of the
bilayer lipids that are broadly distributed in space and time. The dynamics can be
inherently collective due to slowly relaxing structures of the flexible lipids. Both views have been discussed [1] yet a clear distinction remains under debate. How can we formulate the various lipid motions in terms of their types, amplitudes, and rates as characteristic of the molecular dynamics within the possible nanostructures?

### 7.7.1 Rotational modes and inertial averaging

Possibly the simplest extension of well-established concepts for simple liquids or complex fluids is to approximate the molecular motions as those of a rigid body, with discrete rotational modes [105]. The motions of the flexible phospholipid molecules might then be understood by inertial averaging over the internal degrees of freedom. One can assume a single averaged inertial tensor over the various length and timescales considered, that is, corresponding to the molecular size and/or the bilayer thickness. Non-collective molecular lipid rotations and wobble can occur around the principal axes of the motion-averaged inertial tensor. By considering the averaged moments of inertia, the molecular rotations around the principal axes of the diffusion tensor would entail the mean-torque potential due to all the other lipids of the membrane [155]. But what are the internal coordinates for the internal averaging – segments, chains, entire molecules, or collections of molecules? The flip side is that this approximation bypasses the molecular flexibility. As noted earlier, both spin-label EPR [7] and solid-state $^2$H NMR spectroscopy [54, 55] give a profile (flexibility gradient) of the order parameters versus the depth within the bilayer. Entanglements of the membrane phospholipid molecules can occur over the various length scales. Even if we can make a timescale separation, it is still open to question whether the integration should extend over all the internal degrees of freedom of the molecules for a given time interval. Alternatively, one can consider a distribution of length and timescales, which for a continuum approximation essentially brings us back to a simple picture in terms of ODF [1, 97].

### 7.7.2 What is the connection to experimental NMR relaxation data?

For reasons of brevity, the question of molecular rotational modes in lipid bilayers in the liquid-crystalline state will not be addressed here in any detail. For a non-collective molecular model [1, 156, 157], a key prediction is that the modes predict a minimum in the spin-lattice relaxation times ($T_1Z$) versus temperature [1, 158], as shown experimentally for membrane proteins [1, 27, 158]. The relaxation is most efficient when the power spectrum of the stochastic molecular fluctuations (spectral density) is optimally matched to the (coherent) nuclear resonance frequency, which occurs when the inverse correlation time $1/\tau_C \approx \omega_0 = 2\pi\nu_0$ where $\nu_0$ is the Larmor frequency. But if an interpretation in terms of discrete rotational modes applies, why has a minimum in the
spin-lattice relaxation times [158] never been observed at any temperature or frequency for lipid bilayers in the liquid-crystalline state? Evidently a relaxation minimum is found mainly for lipid systems with a large reduction in the configurational degrees of freedom – as in the solid-ordered (so) (gel) state, or when cholesterol is present in the lo state [95, 96] – but not for the liquid-disordered (ld) state.

Arriving at this juncture, we can say the following: consideration of a mean-field potential picture is of course possible. But what do we gain by analyzing the non-collective motions (rotational modes) for individual molecules within the mean field due to all the other molecules of the bilayer? In effect, we interpret our assumption rather than testing its validity. To our knowledge, for lipids in the liquid-crystalline state, no experimental proof for rotational modes of the entire lipid molecules or even portions of the flexible lipid molecules has been obtained, except for the phosphodiester moiety [94], for example, in contrast to microwave spectroscopy of small molecules in the gas phase [159]. Perhaps such a relaxation ($T_1 Z$) minimum is not observed because distinct rotational modes of the lipid molecules – corresponding to a unique inertial tensor that describes the (non-collective) molecular motions – are not present. Alternatively a broad distribution of relaxation modes can exist, consistent with quasi-elastic (harmonic) excitations, spanning the bilayer dimensions down to the segmental size, as we have proposed [1]. By analogy with liquid crystals, we can treat the latter as ODF.

### 7.8 The emergence of membrane elasticity

The above-mentioned findings suggest that for bilayers in the liquid-crystalline state, we can put aside our molecular view for a continuum picture, with a distribution of harmonic or quasi-elastic excitations of the flexible lipids. Because the lipid motions are inherently collective, it is open to discussion whether a picture applies in terms of stochastic modes due to non-collective molecular rotations. The alternative is a composite membrane deformation model, whereby the molecules are entangled, with collective interactions due to their tethering to the aqueous interface. Collective fluctuations in the local ordering of the lipids give a broad continuum of elastic, wave-like disturbances. These order fluctuations explain the frequency dependence of the nuclear spin relaxation rates ($R_{1 Z}$ and $R_2$) by a distribution of correlation times for membrane liquid crystals. Seemingly at short distances, a nematic-like 3D model can approximate the ODF of fluid membranes. For distances much greater than the bilayer thickness, however, a transition to 2D smectic-like order fluctuations of an elastic sheet can occur. A remarkably simple square-law functional dependence of the experimental relaxation rates and order parameter profiles of the acyl groups follows in either the 3D or the 2D limits (Figure 7.3). The underlying explanation is the emergence of bilayer elasticity over the short distances [58]. Invoking the equipartition theorem, with first-order (exponential) (over)damping of the modes, we readily get to the predicted frequency
dependence of the relaxation rates in a continuum approximation. In this way, we find an $\omega^{-1/2}$ frequency dependence for 3D nematic-like fluctuations of the hydrocarbon core; for a 2D elastic sheet, an $\omega^{-1}$ law is obtained, and lastly for elastic fluctuations of a 1D string an $\omega^{-3/2}$ dependence is present [132]. Collective modes emerge on the meso-scale of the bilayer thickness and less, and are connected with the bulk membrane elasticity [151, 160–162] by a distribution of slowly relaxing structures, that we formulate as ODF [60, 97]. Hence, we arrive at a new view of the lipid bilayer as a membrane liquid crystal [58] – albeit, one endowed with physicochemical properties of high biological importance.

7.8.1 Physical significance of the order-director fluctuations

At any rate, the most telling aspect entails the following question: What is the physical significance of the combined NMR order parameter and relaxation approach in terms of the multi-scale lipid dynamics? And how are the order fluctuations related to the corresponding lipid membrane properties? To this end, Figure 7.5 gives us a summary of previous data for the fatty acyl chains of multilamellar dispersions and small vesicles of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) in the liquid-crystalline state [58]. In Figure 7.5a, the square-law functional dependence of the $^2$H $R_{1Z}$ relaxation rates and $^2$H NMR order parameters along the acyl chains is depicted for DPPC multilamellar dispersions. Remarkably, in accord with the equipartition theorem from statistical mechanics, the harmonic excitations extend down to a length scale approaching

![Figure 7.5: Experimental results from NMR spectroscopy indicate the physical significance of bilayer fluctuations. (a) Plot of $^2$H spin-lattice relaxation rates $R_{1Z}$ versus squared order parameters $|S_{CD}|^2$ for acyl segments of multilamellar dispersions. (b) Plot of $^{13}$C spin-lattice relaxation rates $R_{1Z}$ for (CH$_2$)$_n$ resonance against $\omega_{C}^{-1/2}$ for small unilamellar vesicles. Data are summarized for DPPC in the liquid-crystalline (also known as liquid-disordered, ld) state at $T = 50$ °C. Theory indicates that both dependencies arise from relatively slow order fluctuations due to collective bilayer excitations. ODF are due to collective lipid motions and correspond to emergent viscoelastic properties of the lipid bilayer. Figure adapted with permission from Ref Brown et al. [58].](image)
the segmental or atomistic dimensions \[60, 97\]. In addition, the magnetic field dependence (frequency dispersion) of the NMR relaxation rates corresponds to various simplified power laws that manifest the types of fluctuations. As a further example, the \( \omega^{-1/2} \) frequency-dispersion found in \(^{13}\text{C} R_{1Z} \) relaxation studies of small DPPC vesicles is shown in Figure 7.5b. Both dependencies are indicative of the fluid, \( \text{Id} \) phase of membrane lipid bilayers. To all appearances, for harmonic excitations, the integration over the entire bilayer (neglecting the high- and low-frequency cutoffs) explains the simple \( \omega^{-1/2} \) frequency dispersion law \[1\]. By contrast, Figure 7.5b shows the \(^{13}\text{C} R_{1Z} \) rates of liquid paraffins such as \( n \)-Hexadecane are approximately independent of frequency – that is to say, the magnetic field strength – over the entire range is considered.

7.8.2 The microviscosity of a lipid bilayer

Equally important, according to Figure 7.5b there is clearly an enhancement in the relaxation of lipid bilayers versus simple hydrocarbon fluids. The local ordering of the lipids, for example, due to segmental isomerizations, is modulated by additional fluctuations of larger orientational amplitude. Apparently, the contributions from the order fluctuations make the lipid relaxation in the NMR frequency range (kHz–MHz) more effective than for hydrocarbon fluids. In simple physical terms, the enhancement depends on both the frequency, that is, the magnetic field strength, as well as the ordering or mean-square amplitudes of the fluctuations. Slower motions not found in simple hydrocarbon fluids are tied to the collective properties of the assembly. The spectral density due to the internal rapid chain fluctuations extends to relatively high frequencies (correlation times \( \tau_C \approx 5–20 \text{ ps} \)). Extrapolating the relaxation rates to zero ordering or infinite frequency gives us the local contribution to the relaxation rates, which matches liquid hydrocarbon taken as a reference. Consequently, the local microviscosity of the bilayer hydrocarbon core – where a bulk viscosity cannot be measured – amounts to a fluidity of only a few centipoises (cP), as originally proposed \[1\], and supported by the highly influential molecular dynamics simulations of Richard Pastor et al. \[134\].

It follows that collective bilayer excitations emerge over mesoscopic length scales between the molecular and bilayer dimensions, and are important for the lipid organization and protein interactions. A schematic cartoon (Figure 7.6) illustrates the collective excitations that may explain the frequency- and order-dependent relaxation enhancement of membrane bilayers (as drawn by the author) \[58, 131\]. By analogy to nematic liquid crystals \[163\], the collective dynamics are approximated by twist, splay, and bend deformations in the high frequency (or free membrane) limit, or alternatively by smectic undulations involving splay in the low frequency (or strongly coupled) limit \[130\]. Extension of such a continuum approach to distances approaching the acyl segmental dimensions approximates the many-body problem encapsulated by molecular dynamics simulations.
7.9 Return to the future

Looking back, what made McConnell such an exemplary scientist and an inspiration to his many admiring colleagues entailed the interplay of experimental approaches with fundamental principles. By focusing on self-taught concepts and useful applications of physical chemistry, he emphasized simplifying generalizations. Following his approach, NMR spectroscopy allows us to explore the multi-scale structure and dynamics of biomembranes, giving complementary new insights to other biophysical methods. Solid-state NMR not only yields access to the lipid components of membranes but also the proteins [26, 158, 164–167]; and the same is true for spin-label EPR spectroscopy [22, 25, 168–170]. Increasingly, we can expect relaxation times to be combined with molecular spectroscopy [171–173], bearing down on the essential questions of membrane biophysics. Together with theoretical approaches, new experimental innovations bring with them a heightened awareness of how biomolecular structure, dynamics, and function are interrelated. Following the path set forth by McConnell and his colleagues, magnetic resonance approaches will continue to inspire and inform our appreciation of lipid biophysics both now and in the times to come.

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