Atomistic resolution structure and dynamics of lipid bilayers in simulations and experiments

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Abstract.

INTRODUCTION

1.Samuli: Add citations to the introduction Atomistic resolution molecular dynamics simulations of lipid bilayers are nowdays widely used technique to seek answer to various research questions. Typically interactions between other biological molecules (e.g. proteins, drugs, ions etc.) and lipids are studied but sometimes also lipid properties are directly under interest. The questions are often biologically motivated and the atomistic resolutions simulations gives very detailed information which is experimentally unattainable.

When simulations are used in this kind of studies, it is necessary to understand the limitations of the method and also the accuracy of the used model. In the pioneering atomistic resolution lipid bilayer simulations the quality of the simulation respect to reality was measured mainly by comparing the acyl chain order parameters and area per molecule between experiments and simulations. Especially some simulation models repruced these amazingly well which led to the wide usage of these models.

Despite of the success of the models to reproduce the acyl chain properties and molecular density more or less correctly, already early days it was pointed out by comparing simulations to various experiments that the glycerol backbone and choline headgroup order structure may not have been correctly described. However, at the time simulations were very short compared to currently accessible timescles and it was not clear if the molecules had time to sample all the states the model would predict. Also the method to quantitatively measure atomistic resolution molecular dynamics and compare to simulations was not available, thus the real sampling timescales were not known. For these reasons the estimates of the quality of headgroup were inconclusive on the early days of molecular dynamics simulations of lipid bilayers and the issue has gained more attention only very recently.

While the C-H bond order parameters for all hydrocarbon segments are yet the core parameter to quantify the lipid model quality, the area per molecule is quite generally replaced with form factor. The main reason is that the area per molecule is calculated from the scattering data using a model (set of assumptions). Thus, when this value is compared to the value from simulations, the simulations are not compared directly to experiments but to a value which comes from another model (set of assumptions to calculate the area per molecule). For this reason the area per molecule is nowdays replaced by comparison between form factor from simulations and x-ray or neutron scattering.

In this review we discuss the current state of the art methods to compare the atomistic resolution lipid structure and dynamics in simulations to the experiments. The C-H bond order parameters measured with NMR and form factors measured with x-ray or neutron scattering are discussed for structural comparison, and spin lattice relaxation rates for the comparison of dynamics. The main advantages of these parameters are that the experimental techiques are non-invansive, they are measured from multilamellar phase which is practically always present in simulations as well due to periodic boundary conditions and that the compared quantity (order parameter, spin lattice relaxation and form factor) is achieved from the actual experimental data in a robust way. The experimental results from these experimental techniques are also highly reproducible and the measured timescales are appropriate for the comparison to simulations. Also several other experimental parameters and techniques are used to quantify the simulation quality, however, none of these is as robust as order parameters, spin relaxation rates or form factor. The most commonly used other techniques are shortly discussed in the end of the review.

C-H BOND ORDER PARAMETERS AS ATOMISTIC RESOLUTION STRUCTURAL MEASURE

2.This is the first sketch of this section. It is composed from the content in the blog and from the things which came into my mind. A lot of references should be added, the text should polished, things should be added and checked and figures should be improved. However, the main strucutre and idea of the section should be visible.

Here will be described:

How are the order parameters measured.
What is the primary experimental observable.
How accurate are the experimental results.
How order parameter is calculated from simulations.
How accurate are the order parameters from simulations.
What can be learned about the structure when comparing order parameters between experiments and simulations

Definition and properties of C-H bond order parameter

In lipid bilayer systems the order parameter of a hydrocarbon C–H vector is typically defined as

$$S_{\mathrm{CH}} = \frac{1}{2}\langle 3\cos^2\theta - 1\rangle, \quad (1)$$

where the angle brackets denote an ensemble average over the sampled conformations, and θ is the angle between the C–H bond and the membrane normal. The numerical values of order parameters vary between $-\frac{1}{2} < S_{\mathrm{CH}} < +1$ depending on the sampled θ distribution. The motivation for this definition is its connection to the dipolar and quadrupolar splitting measured with ^{1}H-^{13}C NMR techniques and ^{2}H NMR techniques, respectively. The definition has a form of second order lagrangian which arises from fundamental theory of interactions between spin systems [**?**]. This theory gives a connection between structures sampled by lipid molecules and experimental observables through the C-H (or C-D in ^{2}H NMR) bond order parameter. However, the connection between structure and order parameters is not fully uniques since several angle distribution can give the same order parameter. On the other hand, the order parameter can be uniquely calculated from the known angle distribution. Thus, the order parameters can be calculated from the suggested structural model and compared against the experimental values to check the quality of the model but the structural models cannot be directly build fromt the order parameters. Molecular dynamics simulations is very popular techique to study the sampled atomistic resolution stuctures of lipids and other biomolecules and comparison of them to NMR order parameters has been very useful in order to validate simulation results, and also to interpret the experiments.

Order parameters give very detailed information about atomistic resolution structure since the values can be measured with high quantitative accuracy for each C-H bond present in the lipid molecule. In most experiments the absolute value is measured, however, also the sign can measured and is known for several segments. Especially it should be noted that the order parameter might have different value for different C-H bonds in the same carbon and this difference is also detectable in experiments and simulations. In this review we call the phenomenon of unequal order parameters for hydrogens attached to the same carbon *forking* as done also in our recent work [**?**]. This large amount of accurate local information about the structure makes the order parameters the best parameter to evaluate atomistic resolution structure of different models against the experiments; if discprepancy is found in order parameter related to certain bond it is known that the model has problems in the orientation of this specific bond. This gives order parameters a significant advance over, e.g. other accurate NMR obervables like ??. These can be also used to quantify the structural quality, however, in the case of disagreement the problem points in the model are more difficult to localize.

The absolute values of order parameters can be measured by detecting quadrupolar splitting with ^{2}H NMR [1] or by detecting dipolar splitting with ^{1}H-^{13}C NMR [2–5]. The measurements are based on different physical interactions, and also the connections between order parameter and quadrupolar or dipolar splitting are different.

Order parameters from ^{2}H NMR experiments

In the ^{2}H NMR experiments the absolute values of order parameters are connected to the measured quadrupolar splitting $\Delta\nu_Q$ (^{2}H NMR) through the equation

$$|S_{\mathrm{CD}}| = \frac{4}{3}\frac{h}{e^2qQ}\Delta\nu_{\mathrm{Q}}, \quad (2)$$

where e is the elementary charge, Q is the deuteron quadrupole moment and h is the Planck's constant. The parameter q is related to the largest electric field gradient and in practise its value is not known; therefore the static quadrupolar coupling constant $\frac{e^2qQ}{h}$ is defined, and its value measured for different compounds in their solid state. In C-D order parameter measurements for lipids, it is typical to use the value measured for different alkenes, $\frac{e^2qQ}{h}$=170 kHz. The relation between order parameters and quadrupolar splittings then becomes $S_{\mathrm{CD}} = 0.00784\times\Delta\nu_{\mathrm{Q}}$. This relation is useful as many publications report only the quadrupolar splittings. For a review and more accurate description see the work of Seelig [1].

Order parameters from ^{13}C NMR experiments

In ^{1}H-^{13}C NMR experiments the dipolar splitting $\Delta\nu_{\mathrm{CH}}$ is measured. This is then related to the effective dipolar coupling d_{CH} through a scaling factor depending on the pulse sequence used in the experiment [2–5]. The effective dipolar coupling d_{CH} is connected to the absolute value of order parameter through equation

$$|S_{\mathrm{CH}}| = (\frac{D_{\max}}{2\pi})^{-1}d_{\mathrm{CH}}, \quad (3)$$

where $D_{\max} = \frac{\hbar\mu_0\gamma_h\gamma_c}{4\pi\langle r_{\mathrm{CH}}^3\rangle}$. r_{CH} is the C-H distance, μ_0 is the vacuum permittivity, and γ_h and γ_c are the gyromagnetic constants for ^{1}H and ^{13}C nuclei. In contrast to Eq. 2, all the parameters in Eq. 3 are in principle known. However, for the internuclear distance only the average r_{CH} is known, not the third moment r_{CH}^3. For this reason values between 20.2-22.7 kHz are used for $\frac{D_{\max}}{2\pi}$ depending on the original authors [2–5, 29**?**].

Quantitative accuracy of experimental order parameter values

It must be stressed that ^{2}H NMR and ^{13}C NMR are fully independent experiments since the deuterium quadrupolar split-

ting $\Delta\nu_Q$ and the dipolar splitting d_{CH} are different physical observables. In addition, the prefactors connecting the observables to the order parameter (Eqs. 2 and 3) are independently measured. Further independent experiments are performed With ^{13}C NMR by measuring the ^{1}H-^{13}C dipolar couplings using different pulse sequences [2–5] when the connection between dipolar splitting d_{CH} and effective dipolar coupling d_{CH} is different.

The order parameters measured with different techniques are compared by several authors, see Table 1 by Gross et al. [3], Table 1 by Dvinskikh et al. [4], Table 1 and Fig. 3 by Ferreira et al. [5] and Fig. 2 and related discussion by Botan et al. [**?**]. The absolute values of order parameters measured with different techniques by independent reasearch groups are in good agreement. By comparing different literature values for the choline headgroup and glycerol backbone Botan et al. suggest that order parameters would be known with the accuracy of ± 0.02 for purified PC lipid bilayer samples. The lower order parameter reported in some studies [**?**] were suggested to arise from lower experimental accuracy. Based on this comparison Botan et al. suggested sweet spots where choline and glycerol backbone order parameters should fall in the simulation models, see Fig. 7. For acyl chain such systematic comparison of values from different sources has not been done. It seems that for some segments the 0.02 accuracy would could not be achieved with similar comparison. However, in general also the acyl chain order parameters from different sources are also in good agreement as demonstrated, e.g. by Ferreira et al. [5], see Fig. 2.

The measurement of quadrupole and dipolar splittings are relatively accurate, especially for quadrupolar splitting. HOW ACCURATE EXACTLY? Thus the quantitative accuracy of measured order parameters is largely affected by the accuracy of the prefactor connecting the measured splittings to the order parameters (Eqs. 2 and 3). Since the accuracy of these prefactors is single technique is not easy to estimate. However, comparing the order parameters measured with different techniques based on different physical interactions the accuracy of the prefactors can be estimated. Since the order parameters from different techniques are almost always within ± 0.02 this can be considered to be the quantitative accuracy of the measurements.

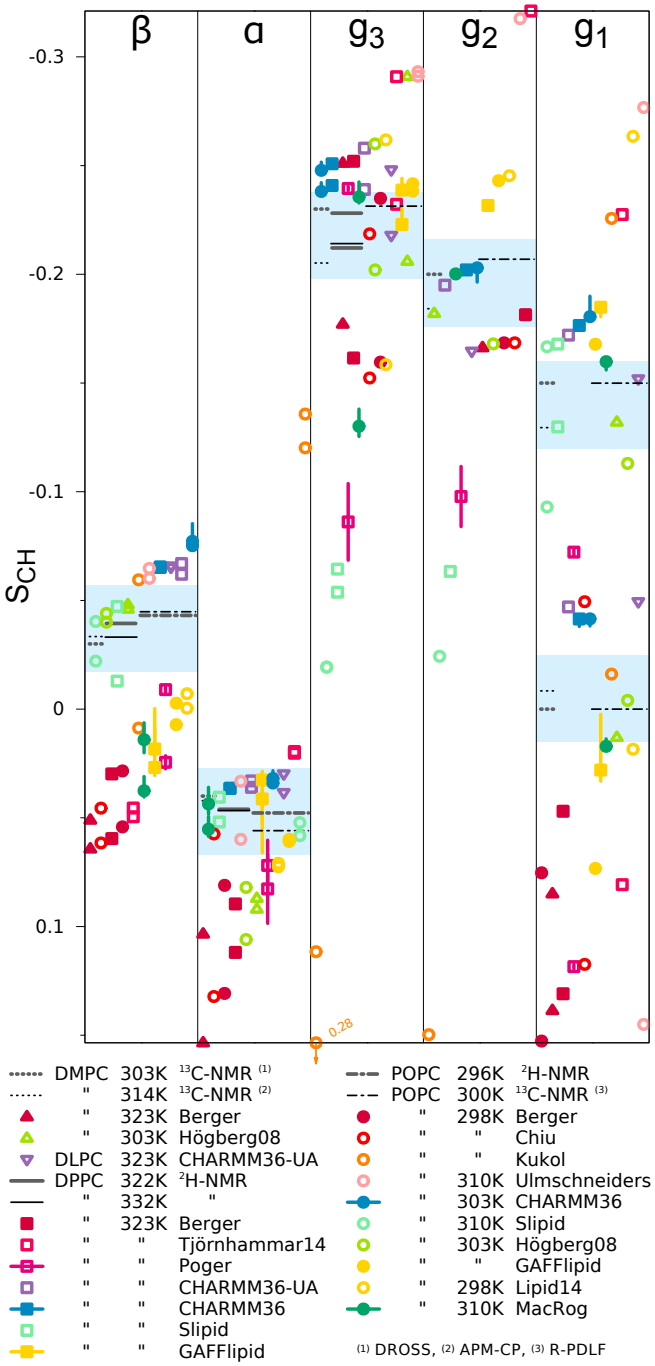

FIG. 1: Order parameteres from simulations and experimental values from literature for glycerol and choline groups collected by Botan et al. [**?**]. The experimental values were taken from the following publications: DMPC 303 K from [3], DMPC 314 K from [4], DPPC 322 K from [6], DPPC 323 K from [7], POPC 296 K from [8], and POPC 300 K from [5]. The vertical bars shown for some of the computational values are not error bars, but demonstrate that for these systems we had at least two data sets (see Table 1 in Botan et al. [**?**]); the ends of the bars mark the extreme values from the sets, and the dot marks their measurement-time-weighted average. The interactive version of this figure is available at https://plot.ly/∼HubertSantuz/72/lipid-force-field-comparison/.

Qualitative accuracy of experimental order parameter values

When considering the change of an order parameter with a changing external condition (hydration, ion concentration, etc.), the prefactor can be considered to be unchanging. Therefore, the change in a given order parameter, when measured by the same people with the same technique and equipment, can be determined in a much higher accuracy than the absolute value of the order parameter. We refer to this as the relative accuracy. It is determined by the accuracy of the splitting measurement. Especially the very high spectral resolution of the ^{2}H-measurements allows the measurement of very

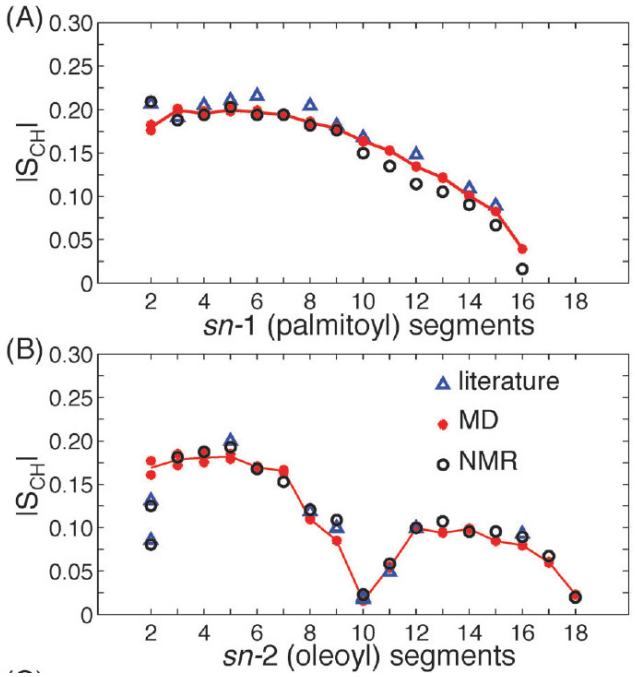

FIG. 2: Picture adapted from [5]. Order parameter magnitude $|S_{\rm CH}|$ vs. carbon segment number for the sn-1 and sn-2 acyl chains of POPC (A and B respectively). Data from fully hydrated POPC at 300 K obtained with ^{1}H^{13}C solid-state NMR (black dots) [5] and MD simulations (red dots) [5], as well as data from 2H NMR (blue triangles) (sn-1 [9] and sn-2 [9, 10] at 300 K).

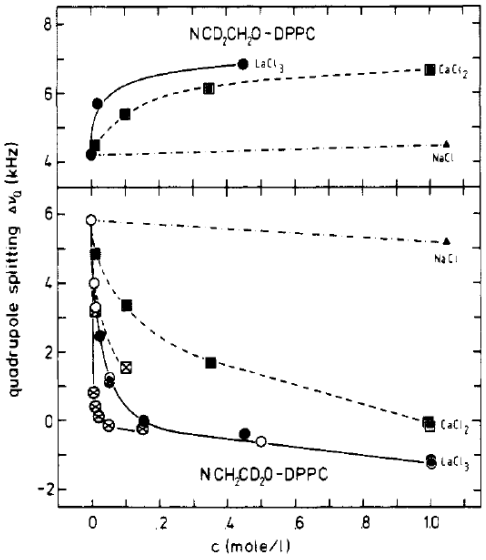

FIGURE 4: Adsorption isotherm of DPPC multibilayers for different types of cations at 59 °C. The quadrupole splittings of DPPC-α- and -β-CD_2 are plotted as a function of ion concentration. Only the size, but not the sign, of the quadrupole splitting can be measured. (Open symbols) 30 mg of lipid was dispersed in 30 mL of buffer and centrifuged to form a pellet; no buffer was used for $LaCl_3$ dispersions (pH ~5); (closed symbols) constant lipid concentration of 70 mM DPPC in buffer (cf. Materials and Methods); (crossed symbols) in addition to $CaCl_2$ and $LaCl_3$ the dispersion contained 1 M NaCl.

FIG. 3: The measured quadrupolar splittings with the explanation in the original caption. Reprinted with permission from Akutsu and Seelig [7]. Copyright (1981) American Chemical Society.

small order parameter changes. Let us exemplify this with a classical experiment by Akutsu and Seelig [7], where the effect of different ions on the quadrupolar splittings of choline headgroup α and β segments was measured, see Fig. 3.

Clearly, the effects of different ions on the quadrupole splittings are differentiable within experimental accuracy in Fig. 3. (These changes were later shown to be consistent with the addition of different charges into the bilayer, and the electrometer concept was introduced to measure the amount of charge incorporated in the bilayer interface [11].) Importantly, when the results of Fig. 3 are transformed to order parameters in Fig. 4, one can see that the numerical changes in $S_{\rm CD}$ are rather small: the y–axis only spans ¡ 0.03 units for β and 0.06 for α.

The resolution of ^{13}C NMR experiment depends somewhat on the pulse sequence used. For example Dvinskikh et al. measured the effect of dehydration in the lipid order parameters with APM-CP pulse sequence, and changes of 0.01 were measurable [12]. Fig. 5 shows the order parameters for α-and β-carbons as a function of dehydration independently measured with ^{13}C NMR and ^{2}H NMR for different PC lipids. All the results are qualitatively similar, that is, they give the same relative change with dehydration.

Signs of order parameters

The absolute values of order parameters are accessible with both ^{2}H NMR and ^{1}H-^{13}C NMR techniques. However, only ^{1}H-^{13}C NMR techniques allow also the measurement of the sign of the order parameter [2, 3, 14]. In older publications that used ^{2}H NMR measurements, only the absolute values of the order parameters (or quadrupolar splittings) were discussed since the sign was not measurable. For the tail C-H vectors, however, the sign was believed to be negative because θ was expected to fluctuate around 90^o leading to negative order parameters [1]. This was later confirmed by using ^{13}C NMR measurements [2].

The signs of the ^{13}C-H order parameters have been measured by at least two different techniques and groups: Hong et al. first measured eggPC [2] and then DMPC [14]; later Gross et al. used a different NMR technique to measure DMPC [3]. These experiments are in good agreement with one another and report negative order parameters for almost all the segments, only α and γ are positive. Furthermore, it seems that the signs of choline headgroup and glycerol backbone order parameters [2, 3, 14] and their absolute values [5, 15] are practically unaffected by the acyl tail contents of the bilayers. Thus, it can be fairly assumed that the signs of order

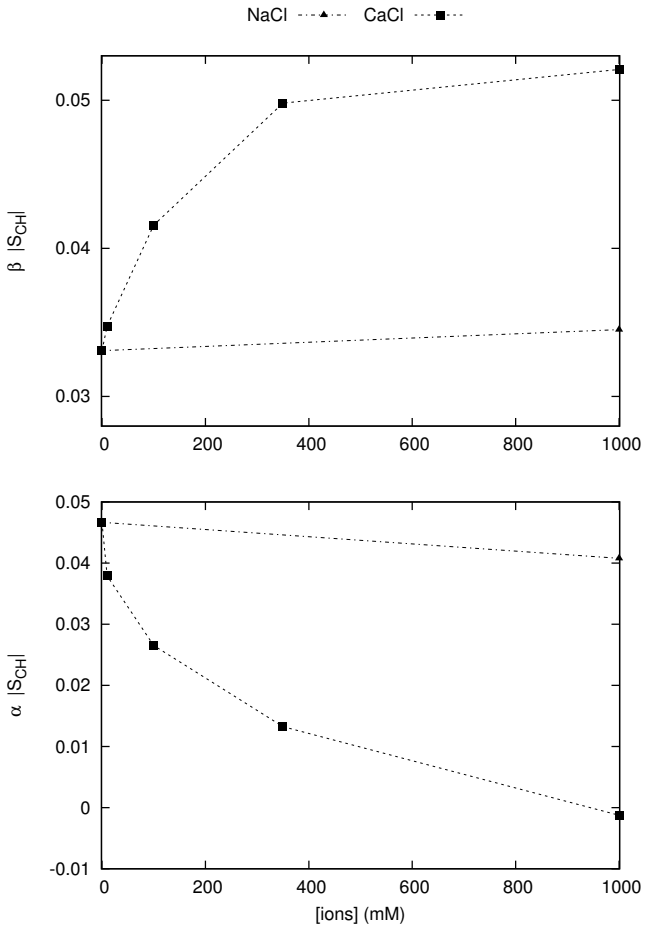

FIG. 4: Same data as Fig.3 but given as order parameters instead of splittings.

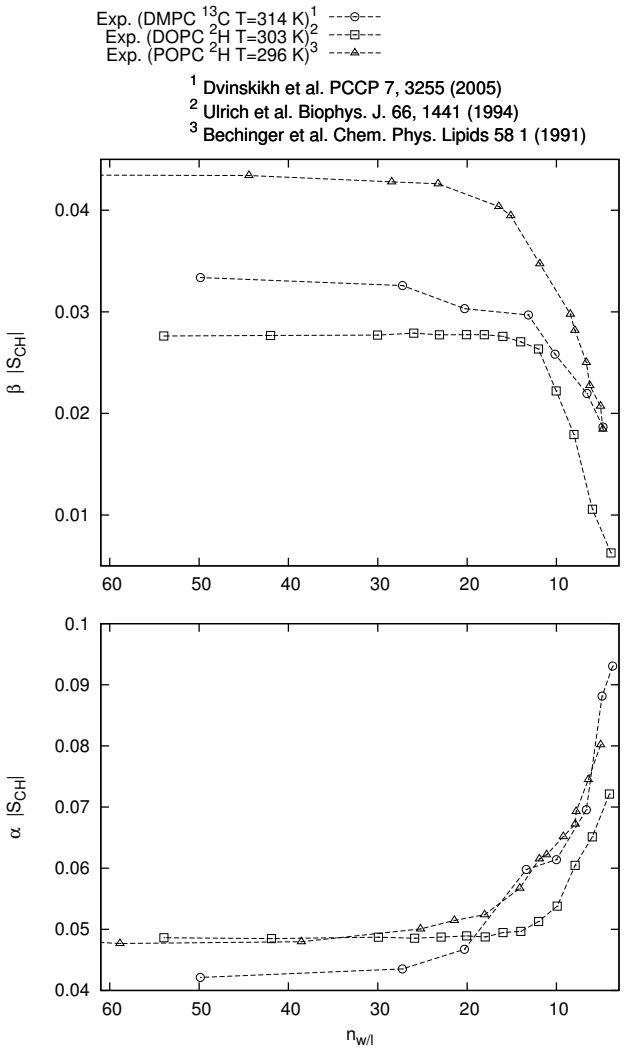

FIG. 5: Dehydration changes on α and β order parameters measured with different methods. The data taken from Dvinskikh et al. [12], Ulrich et al. [13] and Bechinger et al. [8] Same data as Fig.3 but given as order parameters instead of splittings.

parameters for all PC lipids in bilayers are the same. On the other hand, the positive signs reported for g_1, g_3 and C_2 in acyl chain from ^{2}H NMR measurements for DMPC bicelles by Aussenac et al. [16] has generated some confusion in comparison between simulations and experiments [17]. However, Aussenac et al. have not actually measured the signs but used a model to extract the signs.

In conclusion, it seems reasonable to assume that the signs measured with ^{13}C NMR methods [2, 3, 14] can be used for all PC lipids in bilayer, i.e. the signs for almost all the carbons are negative, only α and γ are positive.

Typically when the response of order parameters to varying conditions (ions, dehydration and cholesterol) is measured, only the absolute values are reported. Where clear responses are observed, like for multivalent ions and dehydration, the experiments are done by gradually changing the conditions. The responses of the magnitudes to these changes are systematic, see Figs. 3, 4 and 5. Thus, it is reasonable to assume that also the signs are not suddenly changing. However, it seems that the sign of the α carbon order parameter does change in response to a large amount of bound charge, such as multivalent ions. In this case, the absolute value of the order parameter first decreases to zero and then starts to increase again [18, 19]; the behaviour of the spectra has been nicely illustrated by Altenbach et al. [18], see Fig. 6.

Forking of order parameters

For most CH_2 segments in a fluid phospholipid bilayer, the order parameters of both hydrogens are equal [9, 20**?**]. However, in some cases (e.g., g_1, g_3, and C_2 carbon in the *sn*-2 chain) the two order parameters are not equal; this can be observed with both ^{2}H NMR [9, 15, 21**?**] and ^{1}H-^{13}C NMR

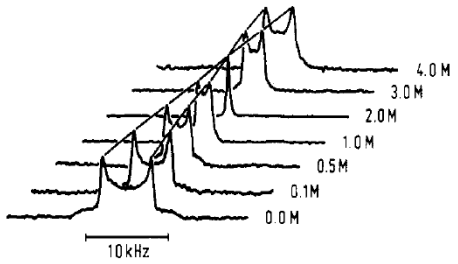

FIGURE 1: ^{2}H NMR spectra of coarse dispersions of POPC bilayers at various $CaCl_2$ concentrations (no NaCl). The lipid was deuterated at the α-segment ($-NCH_2CD_2OP-$). Measuring temperature, 40 °C.

FIG. 6: Quadrupolar splitting $\Delta\nu_Q$ of α of POPC as a function of $CaCl_2$ concentration, related to the order parameter as $S_{\rm CD} = 0.00784 \times \Delta\nu_Q$. We know nowadays that the order parameter of α in the absense of $CaCl_2$ is positive [2, 3, 14]. Thus, the most obvious interpertation for the result is that the α order parameter decreases to zero when $CaCl_2$ concentration reaches 2.0M, and above these concentrations becomes increasingly negative with further addition of $CaCl_2$. Reprinted with permission from Altenbach and Seelig, Biochemistry, 23, 3913 (1984). Copyright 1984 American Chemical Society.

techniques [**?**]. In the present work, to avoid confusion with the dipolar and quadrupolar splittings in NMR terminology, we call the phenomenon of unequal order parameters for hydrogens attached to the same carbon *forking*. Forking has been studied in detail with ^{2}H NMR techniques by separately deuterating the R or S position in CH_2 segment, and the studies show that it arises from differently sampled orientations of the two C–H bonds, not from two separate populations of lipid conformations [15, 21].

Order parameter values in the literature

The acyl chain order parameters are reported extensively in literature for various lipids in multilamellar phase measured with both ^2H NMR and ^{13}C NMR techniques. The values are collected in review articles [22, 23]. However, many of the ^2H NMR studies are measured from lipids with perdeuterated acyl chain(s). In these experiments only numerical values of order parameters are known but the assignment to the specific carbon is not known. However, also order parameter measurements from specifically deuterated acyl chain carbon segments are done [9, 20**?** **?**]. In these experiments the assignment is known but tedious synthesis of specifically deuterated lipids is required.

Also increasing amount of acyl chain order parameters from ^{13}C NMR are reported in literature [5, 22**?** , 23]. The natural abundance ^{13}C NMR experiments gives order parameters simultaneously for all the hydrocarbon segments. However, in these experiments the assignment of spectral peaks is needed and this is tedious for acyl chains due to overlap of spectral peaks. This challenge is overcame with careful experiments and analysis combined to the MD simulations previous specifically deuterated ^2H NMR results [5**?**].

The agreement between acyl chains order parameters assigned by combining these techiques is very good as shown in Fig. 2. Only exception is the simulation results for most models for the C_2 segment in sn-2 chain which has forked order parameters with lower magnitude compared to other segments in the beginnig of acyl chains. This behaviour is systematically observed to all PC lipid bilayers and it is related to the special conformations of lipid molecules [**?**].

Also the order parameters for hydrocarbon segments in glycerol backbone and choline are reported in several ^2H NMR and ^{13}C NMR studies. The values for PC lipids are recently reviwed in the NMRLipids project [**?**]. It should be noted that even the stereospecifity is known for g1 and g3.

There is also increasing amount of order parameter data as a function of changing conditions [**?**]. In the NMRLipids project it was recently demonstrated how this data can be used to check the quality of responses of glycerol backbone and choline in MD models, and also the ion partition. This approach has a lot of potential to elucidate the quality of different simulation models and also to interpret the experiments. In the NMRLipids project the PC lipid dehydration and interactions with ions and cholesterol were compared between different simulation models and experiments. Such comparison can be straighforwardly extended to the systems with experimental data available, e.g. other lipids than PC lipids, to the acyl chain region, to the interactions between, e.g. drugs and poroteins with lipids.

Here we concentrate only directly measured order parameter data from multilamellar samples which correspond typical MD simulation systems in a box with periodic boundary conditions. The order parameters are reported in the measured literature also, e.g., for bicelles [16, 24, 25] or from fits to the relaxation data [**?**]. These order parameters can be often related to the ones from multilamellar systems by using theoretical connections, however, the directly measured order parameters are better for validation of models due to minimal amount of assumptions used to extract the numerical values.

Order parameters from simulations

In molecular dynamics simulations the trajectories of each atom as a function of time are solved by using the Newton's equation of motion and the parameters describing the forces between atoms (force field). Also the thermal motion is included in the simulations, thus the molecules are expected to sample the realistic phase space during the simulation. Since the coordinates of all atoms are known from the simuation trajectory, the order parameters can calculated directly using the definition of Eq. 1 for each hydrocarbon segment.

For simulations models without explicit hydrogen atoms (united atom models), the hydrogen positions can be generated post-simulationally from the positions of the heavy atoms and the known hydrocarbon geometries. Alternatively, the

equations ?? can be used to calculate the order parameters directly. These two approaches are almost identical and give essentially the same results. However, the Eq. **??** is valid only for the case where there is no forking, i.e. order parameters are equal for both hydrogens attached to the same carbon.

In general the order parameters for the acyl chain reported from the same models by different authors are in good agreement in the literature. The exeption is, however, the order parameters for C-H order parameters which are calculated incorrectly by the *g_order* program which is part of the Gromacs package. This discrepancy can be seen for example by comparing the order parameters between ? (calculated with *g_order*) and ? (calculated with Eq. **??** and generating hydrogen positions with *protonate*) for oleyl chain of POPC. In some cases the order parameters are also formally reported to be positive [**?**]. However, in these cases it almost certain that negative values are actually achieved from actual calculations. Most likely explanation for reported positive values are the missing absolute value lines from the figure label, or the confusion arising from the fact that the *g_order* gives actually -S_{CH}. By takig into account the above mentioned and well understood issues, the simulation order parameters for acyl chain in literature are in good agreement with each others and simulations.

Especially the generally good quantitative agreement between experimental and simulated order parameters in acyl chain region for pure lipid bilayers (except for C_2 segment in sn-2 chain) should be emphasized. This means that the atomistic resolution structures sampled by the acyl chains are correct with high probability in the simulations and that the simulations give better and more illustrative description on acyl chain conformations than the traditionally build models based on NMR data. In addition to the quantitatively good agreement in pure bilayers, the changes in acyl chain order parameters as a function of changing conditions are generally reasonable. For example, increase of order parameters as a function of cholesterol concetration and with hydration are reproduced in simulations. In contrast the decrease due to addition of double bonds is also reproduced. It should be noted, however, that the quantitative changes are often not reproduced correctly when compared. This indicates that even though the models reproduce atomistic resolution changes into the correct direction, the changes are often over or underestimated. There is room for improvement in the models in this respect. However, in conclusion, the acyl chain structure and its qualitative changes are generally well described by the molecular dynamics simulations and this is one of biggest achievements of the field, and also one of the most important justification for the usage of such models.

There is more discrepancies in the order parameters and their interpretation reported for glycerol backbone and choline in the literature. In recent publication from the NMRLipids project **??** the results from 12 different models were calculated and compared to the experiments, see Fig. 7. In this study significant differences in order parameters and structures were observed between the models, however, none of

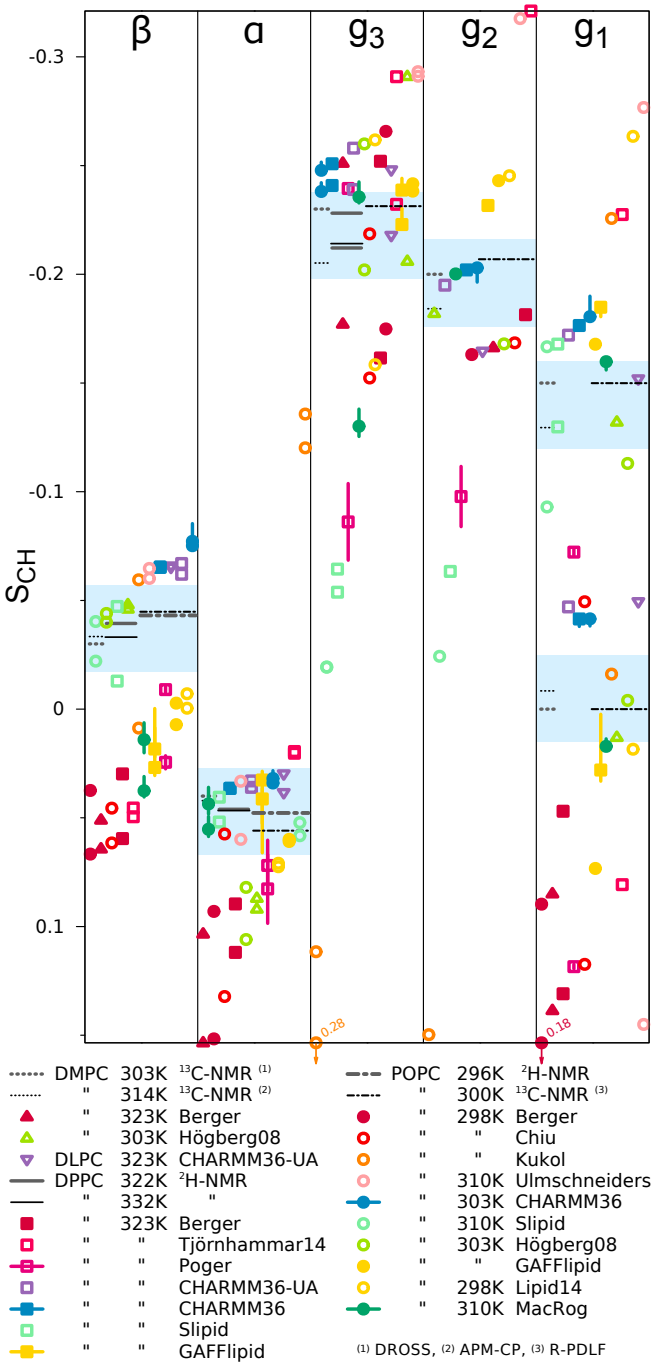

FIG. 7: Order parameteres from simulations with different models and experiments for glycerol and choline groups reported by Botan et al. [**?**]. The experimental values were taken from the following publications: DMPC 303 K from [3], DMPC 314 K from [4], DPPC 322 K from [6], DPPC 323 K from [7], POPC 296 K from [8], and POPC 300 K from [5]. The vertical bars shown for some of the computational values are not error bars, but demonstrate that for these systems we had at least two data sets (see Ref. [**?**]); the ends of the bars mark the extreme values from the sets, and the dot marks their measurement-time-weighted average. An interactive version of this figure is available at https://plot.ly/∼HubertSantuz/72/lipid-force-field-comparison/.

the models reproduced the order parameters within experimental error. This indicates that the simulations models are not in the level that those could be used to study the glycerol backbone or choline structrure, or phenoma related to their strucuture or energetics. Despite of the incorrect structures in simulation models the qualitative responses to the dehydration and charge penetration were qualitatitevly correct. However, the ion partition coefficient was significantly off from experiments in several models. Also the effect of cholesterol on glycerol backbone was overestimated by the most used model. The conclusions from NMRLipids project and the reported numbers differ from some earlier investigations. Some studies have interpreted essentially same simuation results to be in good agreement with experiments [**?**]. This difference in interpretation seems to arise from the underestimated accuracy of experimental order parameters. On the other hand, some studies report different order parameter values than Botan et al. for the same models [**?**]. However, in these studies the order parameter signs and forking are not carefully analyzed which probably explains the differences. It should be noted that all the data and analysis by Botan et al. is openly available, so their calculations can be carefully re-examined, which is not the case for other studies.

The statistical error estimates of simulated order parameters are systematically small. The error of the mean calculated averaging ove time blocks [**?**], independent simulations [26] or different lipids [**?**] all give maximum error $\sim \pm 0.01$.

It has been pointed out recently that the sampling of individual dihedral angles might be very slow compared to the typical (100 ns) simulation timescales [27]. After 200 ns, however, even the slowest rotational correlation function of a C–H bond (g_1) reaches a plateau (S^2_{CH}) in the Berger model [28]—and, notably, the dynamics of this segment have been shown to be significantly slower in simulations than in experiments [29]. In practise, due to averaging over different lipid molecules, less than 200 ns of simulation data should be enough for the order parameter calculation; if the sampling within typical simulation times is not enough for the convergence of the order parameters, then the simulation model in question has unphysically slow dynamics.

Interplay between simulations and NMR order parameters: Validation and interpretation

In the above sections we review the accuracy and availability of NMR order parameters in simulations and experiments. We have concluded that the order parameters for each segments of phospholipids in lipid bilayer are achievable with high accuracy from simulations and experiments, and also lot of these values are already available in the literature. The experimental order parameter give very detailed and local information about the orientations sampled by each C–H bonds in the lipid bilayer system. However, the structure cannot be resolved only by using experimental order parameters since several θ angle (in Eq. **??**) distributions produces the same order parameter and not all segments of lipid molecule contains C–H groups (e.g. phosphate). Historically several modeling techniques have been used to suggest structures which reproduce the experimental order parameters, thus being realistic candidates for the atomistic resolution structure of lipids [**?**]. This has turned out to be extremely difficult task and unambigous solution has not been reached, although some details are agreed [**?**].

The atomistic resolution molecular dynamics simulations of lipid bilayers gives automatically the atomistics resolution structure of lipids sampled in a bilayer. Since the order parameters can be calculated from these simulations with high accuracy, those can be compared to the experimental values. If the order parameters agree within experimental error, the simulated structures are realistic and can be considered as an interpretation of experimental numbers. It should be noted, however, that even if the order parameters would be reproduced, it does not, in principle, guarantee that the structures are correct. Further test can be done by using further dipolar coupling [**?**] and scattering data, see Section **??**. If the simulated order parameter do not fit inside the experimental error bars, the simulation do not sample correct atomistic conformations and the simulation methdology and model should be reconsidered.

As discussed in the previous section, many (if not all) simulations models have serious difficulties to reproduce the glycerol backbone and choline headgroup structure and energetics, their response to the cholesterol and ion concentrations. This indicates that the reliability of most simulation results reported in the literature regarding these issues are not reliable and that the modelling of these parts should be paid more effort before the quantitative structural intepretation becomes possible. Despite of the problems, the qualitative response of headgroup to the dehydration and penetrating ions was correct which confirmed the connection between increasing choline α and β order parameters and increasing P–N vector angle respect to the membrane normal [**?**] suggested previously in [**?**].

As also discussed in the previous section the simulation methods have been much more successfull in describing the acyl chain region of lipid bilayer. Almost all simulation models are able to reproduce acyl chain order parameters with the very good accuracy, exception being the C_2 segment in the sn-2 chain. Also the changes induced in acyl chain region are reproduced well, at least qualitatively. Thus the sampled acyl chain structures given by simulations can be considered to be reasonable interpretation for experimental acyl chain order parameter measurements.

This is a significant advancement to the traditional structural models build based on the fittings to the order paramters. The dynamical visualization of simulation trajectory immediately reveals very dynamical nature of acyl chains, rapidly sampling large amount of different conformations (for dynamics see the Section **??**). These dynamical visualizations gives significantly better intuitive understanding of dynamical nature of lipid bilayers compared to the static ones from tradi-

tional models. Since the lipid bilayers can be considered as a simplistic models for cell membranes and other biological lipid layers, this understanding has significant impact on biophysics and biochemistry.

In addition, the simulations have been useful in understanding the effect of changes induced in the acyl chain region. For example, the decrease of order parameters due to double bonds has been reproduced in several simulations models with very good or decent accuracy [**?**]. These simulations has been then used to shown that the double bonds induce more disordered and fluid bilayer due to the more flexible dihedral potentials next to the rigid double bonds [**?**]. In this respect the simulations showed significant advancement compared to the traditional approaches [**?**]. Also, increased acyl chain order and related thickening of lipid bilayers due to addition of cholesterol is generally reproduced by simulations giving also intuitive visualizations for these effects [**?**]. However, in this case the strength and molecular details of this effect may not fully agree with experiments in all models indicating that there is still room for iprovement in lipid-cholesterol interactions in lipid models [**?**].

Simulation studies have also predicted changes in acyl chain region which are not yet experimentally confirmed, e.g. disordering induced by the lipid oxidation [**?**]. A new experimental data can confirm if these predictions can be verified. In contrast to the glycerol bakcbone and headgroup [**?**], serious simulation artefacts in the acyl chain region in the state of the art simulation models are not reported.

In conclusion, the detailed comparison of atomistic resolution structures of lipids in bilayer between simulations and reality has revealed that the acyl chain region is generally well described in simulations and has provided new information about lipid structures. In contrast, significant problems in glycerol backbone and headgroup region are observed which has led to questionable conclusions [**?**].

C-H BOND ROTATIONAL DYNAMICS FROM SPIN RELAXATION RATES AND SIMULATIONS

Here will be described:

How the rotational dynamics measured by using NMR relaxation experiments.
How the relaxation experiments are connected and compared with simulations.
What can be learned and what has been learned about the rotational dynamics from the comparison between spin relaxation and simulations

3.This is quite straightforward to write for me and there is quite good support from our recent work [29]. I will write the first version as soon as I can.

Definition and properties of rotational autcorrelation function

The second order auto-correlation function for the reorientation of the C–H chemical bond axis is defined as [**?**]

$$g(\tau) = \langle P_2[\vec{\mu}(t) \cdot \vec{\mu}(t+\tau)]\rangle, \tag{4}$$

where P_2 denotes the second Legendre polynomial, $P_2(\xi) = 1/2(3\xi^2 - 1)$, $\vec{\mu}(t)$ is the unitary vector having the direction of the C–H bond at time t, and the angular brackets denote a time-average. This autocorrelation is usually chosen to describe the C–H bond rotational dynamics since it is connected to the experimentally measurable spin relaxation rates through its Fourier transformation called spectral density

$$j(\omega) = 2\int_0^\infty \cos(\omega\tau)g(\tau)d\tau. \tag{5}$$

In this review we focus only on experiments measured from multilamellar samples with randomly oriented sheets, hus only the second order auto-correlation function is needed [**?**].

In randomly oriented multilamellar samples the auto–correlation function of bond orientations always decays to zero with long enough time scales. However, the relaxation timescales can be divided to two distinct timescales. First the relaxation processes shorter than microsecond timescales occurs when lipid molecules are reorienting in the lipid bilyer but are not essentially moving between lipid bilayer regions with different orientations. Then with larger than microsecond timescales the movement between differently oriented bilayer regions decays the rotational correlation function to zero. In addition, MAS experiments the sample spinning causes lead orientaitional relaxation in kHz region. The full auto–correlation decaying to zero is illustrated in Fig. 8. Due to the timescale separation the correlation function can be written as [**?**]

$$g(\tau) = g_{\mathrm{f}}(\tau)g_{\mathrm{s}}(\tau); \tag{6}$$

$g_{\mathrm{f}}(\tau)$ describes the decay of $g(\tau)$ due to fast molecular motions and $g_{\mathrm{s}}(\tau)$ contains the contribution from slower motions

$$g_{\mathrm{s}}(\tau) = e^{-\frac{\tau}{\tau_{\mathrm{s}}}}\left[\frac{2}{3}\cos(\omega_R\tau) + \frac{1}{3}\cos(2\omega_R\tau)\right], \tag{7}$$

where τ_s is a correlation time due to slower isotropic molecular motions originating from the diffusion between bilayers with different orientations of their principal symmetry axis, and the cosine terms are the contribution from magic angle spinning of the sample, rotating at $\omega_{\mathrm{R}}/2\pi$ cycles per second [**?**], typically in the kHz frequency range.

The order parameter measurements with ^{2}H NMR and ^{13}C NMR measure the bond order after the relaxation of rotational motion inside the bilayer plane but before the relaxation between different bilayer orientations, as illustrated in Fig. **??**. In typical molecular dynamics simulations with peri-

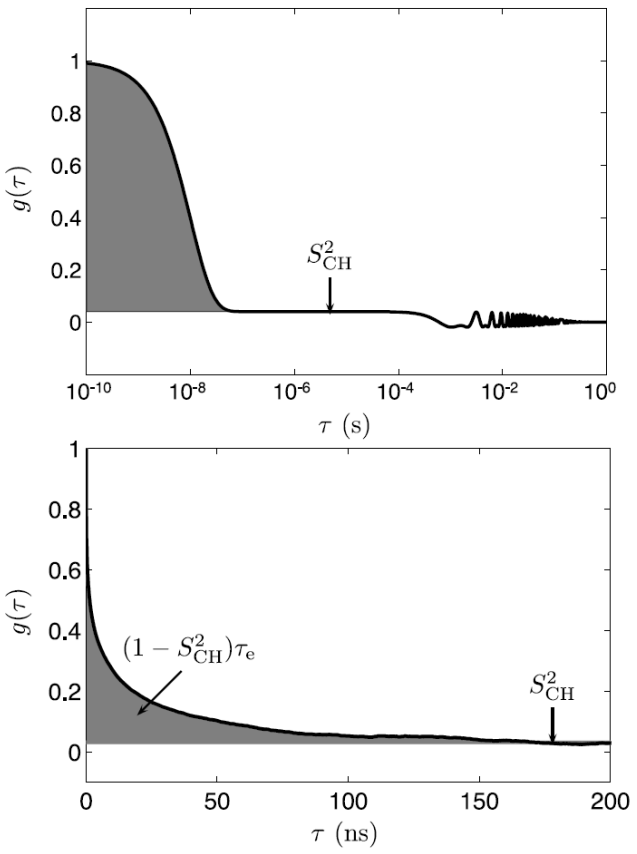

FIG. 8: (Top) Illustration of the auto-correlation function g() and effective correlation time e for a CH bond in a lipid or surfactant bilayer. Magicangle spinning with the frequency R leads to oscillations of g() at the frequencies R and 2R. The slow correlation time s gives the final decay of g() towards zero. (Bottom) Example of g() from a united-atom MD simulation of a POPC bilayer in excess water, illustrating the decay towards S2 CH. The effective correlation time e is equal to the area in gray scaled by (1 S2 CH)1.

odic boundary conditions the lipid molecules are restricted to single bilayer orientation and also the timescales are currently typically below microsecond. In these simulations the auto–correlation function in Eq. **??** decays to the square of order parameter in Eg. **??** in bilayers with planar symmetry, i.e. no microscopic phase separation of defects present. Also this is illustrated in Fig. **??**.

The rotational correlation function describes how long does it take for a single molecule on average to sample the conformations. The effective correlation time

$$\tau_e := \int_0^\infty \frac{g_{\mathrm{f}}(\tau) - S^2_{\mathrm{CH}}}{1 - S^2_{\mathrm{CH}}} \mathrm{d}\tau \qquad (8)$$

can be used as an intuitively useful single parameter to describe this time. The larger this parameter is, the longer it takes in average to sample the conformations related to the bond. With this definition the area between the correlation function and its pleateau becomes $(1 - S^2_{\mathrm{CH}})\tau_e$.

Detecting C–H bond dynamics experimentally

The most used parameter to detect the C–H bond dynamics experimentally in time scales comparable to simulations are the spin-lattice relaxation rates R_1 from deuterium labels and ^{13}C. R_1^C measured from ^{13}C is connected to the spectral density (Eq. 5) through the equation

$$R_1^C = \frac{D^2_{\mathrm{max}} N_{\mathrm{H}}}{20}\Big[j(\omega_{\mathrm{H}}-\omega_{\mathrm{C}})+3j(\omega_{\mathrm{C}})+6j(\omega_{\mathrm{C}}+\omega_{\mathrm{H}})\Big], \quad (9)$$

where ω_{C} and ω_{H} are the Larmor angular frequencies of ^{13}C and ^{1}H respectively, N_{H} is the number of bound protons and $\frac{D_{\mathrm{max}}}{2\pi}$ ≈22 kHz as in section **??**. R_1^D measured from ^{2}H is connected to the spectral density (Eq. 5) through the equation

$$R_1^D = \frac{12\pi^2}{40}\left(\frac{e^2qQ}{h}\right)^2\Big[j(\omega_{\mathrm{D}}) + 4j(2\omega_{\mathrm{D}})\Big], \qquad (10)$$

where $\frac{e^2qQ}{h}$=170kHz as in the case of order parameters in section **??**.

Also the model free approach to measure the effective correlation time (Eq. 8) was recently introduced [29]. The method is based on the combination of experimental order parameter S_{CH}, spin-lattice relaxation rates R_1 and the transverse magnetization under a spin lock pulse $R_{1\rho}$ with measured appropriate nutation frequency.

Analyzing C–H bond dynamics from simulations

As in the case of order parameters, the auto–correlation function for each C–H bond can be calculated directly from simulations using the definition in Eq. **??** since the trajectories of each atom is known as a function of time. As in the case of order parameters the positions of hydrogens can be determined for united atom models based on heavy atom positions and assuming tetrahedral configurations. Usually in the correlation function calculation all the available time intervals from the simulation data are used and the average over those and all molecules is taken. However, since the amount of data decreases when the the time interval approaches the total length of the simulation, usually the largest time interval used is the half of the total simulation length, for more details see **??**.

To calculate the experimentally measurable spin lattice relaxation times, the spectral density (Eq. 5) must be first calculated. In principle, this could be done using numerical fourier transformations techniques, however this often leads to unneccessarily large fluctuations. Instead, commonly used apporach is to fit analytical functional form to the calculated auto–correlation function and then use analytical Fourier transform of the fitted function. Most commonly the sum of ? or more

exponentials is used as a fitting function but also streched exponential has been used. Numerically the functional form of the fitting function should not matter as long as the fit is good, however, theoretically the correct correlation function form to describe the modes of physical motion can be debated. It is clear from correlation functions from simulations that one exponential is not enought to produce a good fit while ? gives a reasonable fit. This is not surprising since more than one relaxation timescale is definitiely expected to be present in lipids in bilayer.

After the fitting the analytical form of the spectral density predicted by simulations is available. Then its values can be calculated at the required larmor frequency values and substituted to Eqs. 10 and **??** to get the R_1^C and R_1^D. The value of the effective correlation time can be calculated directly from the interated area below the correlation function, see Fig. **??** or from Eq. ??.

Interplay between simulations and NMR spin lattice relaxation times: Validation and interpretation of dynamics

The experimentally observable spin lattice relaxation parameter mentioned above (R_1^C, R_1^D and $R_{1\rho}$) are connected to the actual molecular dynamics through the spectral density (Eq. 5) which is the Fourier transformation of the autocorrelation function 4. The spin lattice relaxation rates depends spectral density values only with certain larmor frequecies as seen from Eqs. 10 and **??**. In experiments the Larmor frequency depends on the external magnetic field of the used spectrometer. Thus, with a regular NMR spectrometer only a single 10 and **??** values can be measured. These will only give information about the size of the spectral density close to the used Larmor frequencies. However, to detect the whole rotational correlation function one should have information with all relevant larmor frequency values. The single spin lattice relaxation rates only give an estimate how much of the dynamical prosesses are present with the timescales roughly with inverse of Larmor frequency. Even the qualitative changes in dynamics are difficult to detect by measuring single relaxation rate values since the increase (decrease) of the value only indicates the increase (decrease) of relative significance of the relaxation with the detected timescales. If the general dynamics gets slower or faster depends what happens to the significance of relaxation processes with faster and slower timescales which are not detected by measuring single relaxation rates.

Several experimental and theoretical apporaches has been use to address this issue. Temperature dependence of spin relaxation rates have been measured to analyze the relative molecular rotational relaxation. Spectrometers with different magnetic field strenghts have been used to measure points with several Larmor frequencies. Models are fitted to the spin relaxation data. The effective correlation time is measured which gives a quantitative measure of the general rotational dynamics. Also MD simulations have been succesfull in interpretation of the measurements of the effect of double bonds on molecular dynamics.

Comparison between spin lattice relaxation rates measured with NMR and calculated from MD is also used to validate the correctness of the dynamics in the simulations. The comparison in the early simulations revealed that there are dynamical time scales present in simulations are realistic [**?**]. Later on the comparison between simulations and dispersion data indicated that the simulation dynamics more or less agrees with experiments for some carbons while there is room for improvement for others [**?**]. However, due to the complicated connection between spin lattice relaxation rates and molecular dynamics it is difficult to conclude from these comparison if the dynamics is too slow or fast in the simulations. This issue can be clarified by measuring the effective correlation times with recently introduced methods [**?**]. The comparison between effective correlation times from experiments and simulations reveals that the rotational correlation dynamics is too slow for the glycerol backbone and choline in Berger model. However, this is not very surprising since the Berger model is also sampling wrong configurations for these segments. However, the experimental data and similar comparison would be useful for models sampling more realistic strucutres, e.g. CHARMM36 which dynamics do not fully satisfy the dispersion data.

STUCTURE FACTORS FROM SCATTERING AND SIMULATIONS

For this section I would be more than happy for some help

Here will be described:

How are the form factors are measured.
What is the primary experimental observable.

On these questions I do not know the answer and it is not exactly clear from where I can find the answers. More specifically:

4.Which is the experimental quantity that the scattering machinary exatcly puts out?
How the form factor is determined from the experimental observables?
Which assumptions are needed here?
There is already some discussion about this in the blog by Peter Heftberger and Georg Pabst, but any kind of information from full exaplanation with citations to the hints of relevant literature are helpful here. The more detailed discussion can be found at: https://github.com/NMRLipids/NMRLipids_V-Review/issues/1

How accurate are the experimental form factors.

5.Has this been discussed in the literature already? Any kind of information from full exaplanation with citations to the hints of relevant liter-

ature are helpful here. The more detailed discussion can be found at: https://github.com/NMRLipids/NMRLipids_V-Review/issues/2

How the form factor is calculated from simulations and compared to experimental ones.

6.As far as I have understood, the form factor is simply a Fourier transform of electron density. I have some quick and dirty scripts to calculate those in the NMRLipids III repository: https://github.com/NMRLipids/NmrLipidsCholXray/blob/master/scratch/ffTest.m https://github.com/NMRLipids/NmrLipidsCholXray/tree/master/scratch/ffTest **However, I have not been able to install the SIMtoEXP program (**http://link.springer.com/article/10.1007%2Fs00232-010-9254-5**) so I have not been able to check my script against the standard method. This should be straighforward issue and should become clear once I check the details. Anyway, any kind of information from full exaplanation with citations to the hints of relevant literature are helpful here. The more detailed discussion can be found at:** https://github.com/NMRLipids/NMRLipids_V-Review/issues/3

How accurate are the calculated form factors from simulations.

7.I think that from statistical point of view accuracy is quite high, however I am not sure about the effect of undulations etc. Any kind of information from full exaplanation with citations to the hints of relevant literature are helpful here. The more detailed discussion can be found at: https://github.com/NMRLipids/NMRLipids_V-Review/issues/4

What can be learned about the structure when comparing the form factors between experiments and simulations

8.I have thought that if the form factor is reproduced by the simulation, the electron density profile should be reasonable. However, since some people are tuning the peak highs for better agreement, I am not sure. There is also some connection to the thickness. There is already some discussion about this in the blog with Peter Heftberger and Georg Pabst. Any kind of information from full exaplanation with citations to the hints of relevant literature are helpful here. The more detailed discussion can be found at: https://github.com/NMRLipids/NMRLipids_V-Review/issues/5

CONCLUSIONS

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