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Application of pressure-modulated differential scanning calorimetry to the determination of relaxation kinetics of multilamellar lipid vesicles

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Abstract

We report an extension of the recently published PMDSC method that permitted synchronous determination of heat capacity and expansibility when using slow, defined pressure formats in a DSC scan. Here we applied continuously opposing pressure changes that are fast compared to the time constants of the DSC instrument to study relaxation kinetics of phospholipids. Investigations of multilamellar vesicles of DPPC or DSPC in water revealed for both lipids relaxation times of about 30 s at the maximum of the main transition peak and about 15 s at the maximum of the pretransition. The relaxation times in the transition range are proportional to heat capacity of main- and pretransition. The molecular origin of the relaxation processes appears to stem from pressure-induced water fluxes between the interbilayer region and the bulk water phase.

1. Introduction

The mechanistic and thermodynamic properties of phase transitions of lipid vesicles have been in the focus of a large variety of studies because of their extreme cooperativity and their potential relevance to biological membranes [1,2]. A significantly smaller number of investigations has been addressed to unravelling the kinetics of these transitions although knowledge of rates and relaxation times is of comparable importance for understanding the microscopic events that determine the changes in macroscopic observables such as heat capacity, expansibility and compressibility. This may be due in part to the experimental difficulties involved in combining pressure or temperature perturbation with the proper observation techniques that permit detection of unlabelled phospholipids. Most frequently optical detection was used based on changes in light scattering and infrared absorption or fluorescence, if corresponding markers were attached. Significant insight has also come from time dependent X-ray studies, as they report directly the time course of the molecular structural changes associated with the cooperative transitions. The problems involved in both temperature and pressure perturbation studies arise to a large extend from the extreme cooperativity of the $P_\beta \rightarrow L_\alpha$ phase transition of MLVs, which leads to half widths of a couple of hundreds of a degree. Such a narrow transition renders it very difficult to perform temperature jumps that end at a defined temperature in the transition range. The situation is somewhat simpler with pressure jumps, if they are applied to a calorimetric setup, where the temperature can be controlled and kept constant to within a couple of thousands of a degree during the measurement. Independently of the observation technique generally relatively large temperature jumps or pressure jumps in the range of 40 to 200 bar have been applied to induce conformational changes of lipids and measure the concomitant relaxation kinetics [3–5].

We describe in the present study an extension of our recently published PMDSC method [6,7]. We demonstrate that application of small pressure perturbations between 0.5 and 3 bar permits quantitative determination of relaxation times of lipid systems in the course of a DSC scan. Such pressure changes constitute no risk to current DSC instrumentation and can be easily implemented.

We chose disteaoryl-phosphatidylcholine, DSPC, and dipalmitoyl-phosphatidylcholine, DPPC, as lipids. The relaxation behaviour of DPPC had been studied before, thus our results could be directly compared, whereas for DSPC no data on the slow relaxation phenomena were available. We also report for the first time relaxation times for the pretransitions of both DPPC and DSPC. It appears that there is a general correlation

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between changes of relaxation times observed for the pre- and main transitions and changes in the response functions [8] such as heat capacity, expansibility and compressibility. This conclusion supports previous results obtained for the main transitions of DMPC and DPPC by Grabitz et al. [9,10].

2. Materials and methods

2.1. Preparation of lipid suspensions

Lipids were purchased from Sigma and used without further purification. Multilamellar DPPC vesicle solutions were prepared in water by dispersing the lipid samples followed by repeated heating in the calorimeter between 25 and 50 °C (heating rate 0.2 K/min until the signal was constant). DPPC solutions of 12.4 mg/mL (16.7 mM) were used. Multilamellar vesicles of DSPC were produced by dissolving weighted amounts of the lipid in a chloroform/methanol mixture (9:1), drying the solution under vacuum and suspending the dry film with water. The resulting vesicle solution was vortexed and repeatedly heated over the transition temperature. The concentrations of DSPC used were in the range of 5 to 16 mg/mL (6.3 to 20.2 mM). Alternatively, DSPC suspensions were also prepared in the same manner as DPPC vesicles. The results obtained with both lipids were independent of the method of sample preparation.

2.2. Differential scanning densimetry (DSD)

Density differences between the lipid suspensions and water were determined with the use of two DMA 602 HT external cells employing a DMA 60 measuring unit in the phase lock loop mode (Anton Paar, Graz, Austria). Temperature of the cells was controlled using a Haake PG20 temperature controller and a Haake F3 thermostatted bath. Temperature was measured with a platinum resistance thermometer placed in a capillary tube in the sample cell compartment. Temperature readings were made employing a Keithley 195A multimeter. Density scans were performed with distilled water in the reference cell and lipid suspension in the sample cell. The instrument constant was determined according to the specifications of the producer using distilled water or air in the sample cell and distilled water in the reference cell. The densities of air and water at the particular temperature of the measurement were obtained from tables in the CRC Handbook of Chemistry and Physics.

Generally the heating rate was 0.1 K/min. Density and temperature data were determined every 0.1 K. Only the pretransition of DSPC was measured using lower heating rates i.e. 0.02 K/min and data were collected every 0.04 K. Heating was stopped at the temperature of data collection, and after the system had been equilibrated the time required for a defined number of oscillations of the vibrating tube was measured. More details on DSD studies can be found in Refs. [10,12–16].

2.3. Differential scanning calorimetry (DSC)

Heat capacity measurements were made with a Nano-Differential-Scanning Microcalorimeter (N-DSC), model 6100 from Calorimetry Science Corporation. The volume of both sample and reference cell is 0.299 mL. The frequency of data acquisition of the instrument is 1/s. The pressure control unit was constructed in collaboration with Jürgen Kröninger of the electronic workshop of the Institute of Physical Chemistry of the University of Münster. A block diagram of the control unit can be downloaded from the Analytical Chemistry website. The noise level of a scan with constant pressure is less than 15 nW and the reproducibility with or without refilling is in the order of 0.4 µcal/K. The maximum pressure is limited to 6.5 bar to prevent damage from the calorimetric cells. The pressure control program provides several formats of pressure change as well as possibilities for waiting times at any pressure.

The pressure jumps were performed between 0.3 and 3.3 bar as well as 0.3 and 0.8 bar with a rate of 0.5 bar/s. In different scans different waiting times between pressure jumps were applied. They ranged from 160 s to 400 s for both lipids to allow for proper relaxation at the respective final pressure. As isothermal measurements are principally not possible with DSC, we approximated isothermicity by using very slow heating rates of 0.001 K/min or 0.002 K/min. The relaxation times associated with the main transition of the lipids were found to be of the order of 30 s. Therefore about 90% of the power change resulting from a pressure jump has been compensated after 1 min. During this time the temperature changes by not more than 0.001 or 0.002 K, respectively, depending on the scan rate. This controlled temperature increase is comparable to the magnitude of the pressure-jump induced temperature fluctuation of 0.002 to 0.01 K reported in Ref. [9] for isothermal calorimetric measurements. In contrast to the small pressure jumps of 0.5 to 3 bar that we apply, large jumps up to 80 bar were used in those studies. They were not applied directly to the calorimeter but to a pressure resistant capillary inserted into the sample cell.

We focus primarily on the effects of negative pressure jumps in our analysis of the pressure-induced relaxation of multilamellar lipid vesicles. Negative pressure jumps shift the transition to lower temperature. As a result, at a given

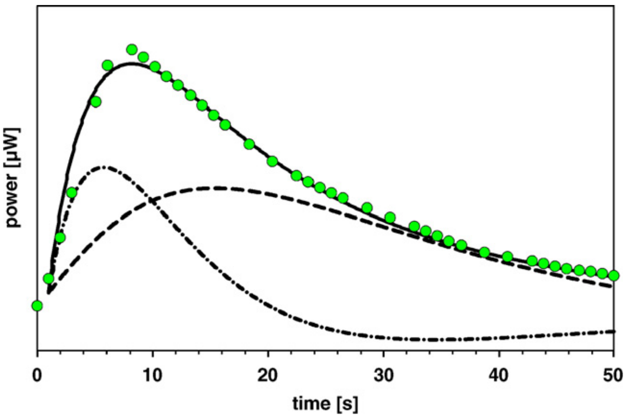

Fig. 1. Deconvolution of the pressure-jump induced calorimetric response signal of a phospholipid suspension. Green dots represent experimental data; solid line: fit of data to Eq. (4); dash-dotted line: water response; dashed line: calculated lipid response. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

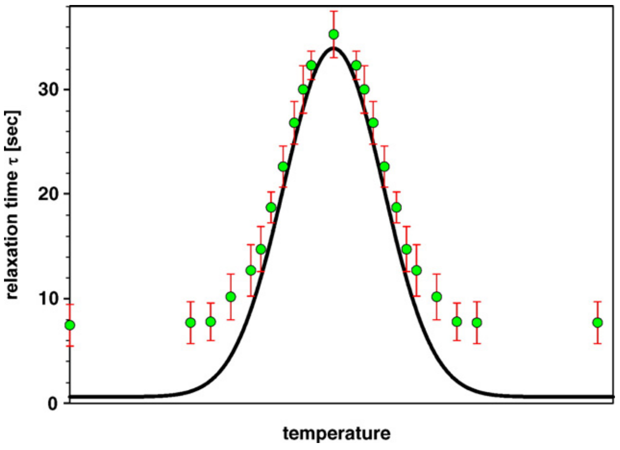

Fig. 2. Comparison between simulated relaxation times and relaxation times obtained from a simple exponential fit to the decaying branch of a calorimetric response curve. The lipid relaxation times were assumed to be Gaussian-distributed (solid peak). The dots with error bars illustrate the relaxation times resulting from the mono-exponential fit to the response curve. The fit approaches on both sides of the peak the instrumental relaxation times.

temperature inside a transition range more lipids undergo conformational changes from L_β or P_β to L_α which requires heat input in the sample cell. Fast heating is easily possible for the instrument. The same fast response is generally not feasible for positive pressure jumps. They induce an increase in the transition temperature which results in increased structure formation of the lipid system and concomitant heat release. Since the calorimeter cannot actively cool during a heating scan, this heat is slow to dissipate and might interfere with the relaxation time measurement. We could show however, that small positive pressure jumps involving only small heat releases, as they for example occur in the pretransitions, provide practically identical time constants as negative pressure jumps.

2.4. Theory

In our analysis of the relaxation time behaviour of lipid vesicles we follow the analysis of Ref. [9]. We assume the following kinetic scheme for the heat released after a pressure jump: lipid $\underset{\tau_{\text{lipid}}}{\overset{k_{\text{lipid}}}{\longrightarrow}}$ water $\underset{\tau_1}{\overset{k_1}{\longrightarrow}}$ cell wall $\underset{\tau_2}{\overset{k_2}{\longrightarrow}}$ detector. The rate constants k_1 and k_2 characterize the instrument response following heat release in form of a Dirac δ pulse. Under these assumptions the normalized instrument response function can be expressed as

$$R_{\text{inst}}(t) = \left(\frac{k_1 k_2}{k_2 - k_1}\right)\{\exp(-k_1 t) - \exp(-k_2 t)\} \tag{1}$$

A derivation is given in Appendix A. Numerical values of k_1 and k_2 can be obtained by fitting pressure-induced experimental relaxation data of water filled cells to Eq. (2).

$$P_{\text{H}_2\text{O}}(t) = P^0_{\text{H}_2\text{O}}[\exp(-k_1 t) - \exp(-k_2 t)] \tag{2}$$

The overall relaxation signal changes, if the sample cell is filled with an aqueous suspension of multilamellar lipid vesicles. Pressure jumps outside the transition range of the lipid will produce response signals that are similar to those of water filled cells. Pressure jumps inside the pre- or main transition cause an additional heat change due to the conformational transition of

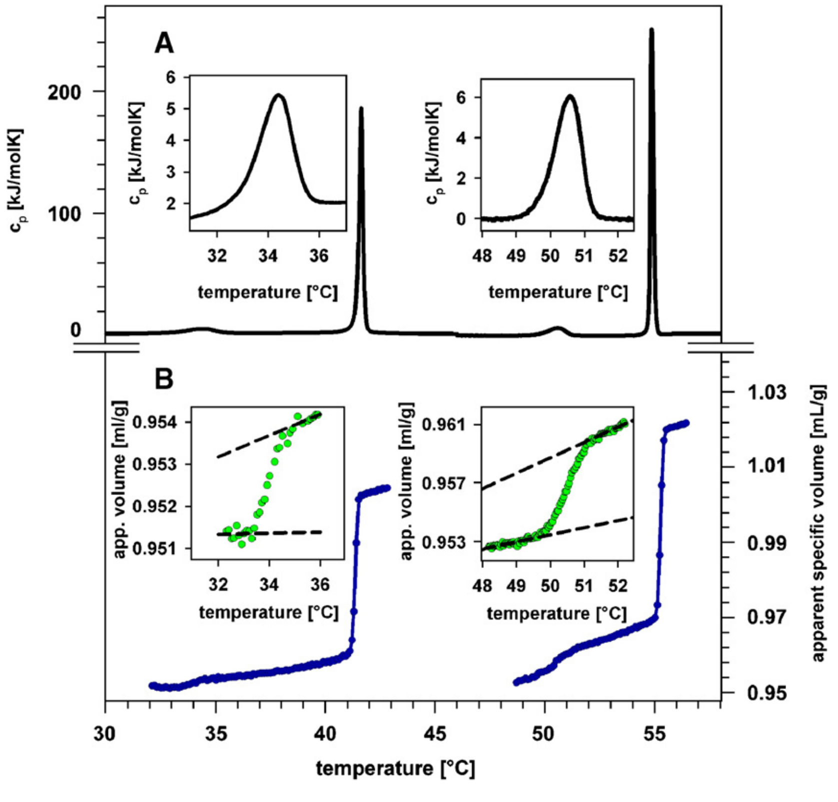

Fig. 3. Heat capacity and apparent specific volume scans of DPPC and DSPC in water. The insets show the magnified pretransitions. A: DSC scans: DPPC concentration: 12.4 mg/mL; DSPC concentration: 15.15 mg/mL. B: Apparent specific volume scans; DPPC concentration: 10.0 mg/mL; DSPC concentration: 15.2 mg/mL.

the lipid vesicles and the reorganization of water of solvation. We assumed that the uptake of heat from the water or release of heat into the water by the lipid system is single exponential.

$$P_{\text{lipid}} = P^0_{\text{lipid}} \exp(-k_{\text{lipid}} t) \tag{3}$$

The recorded power signal $P_{\text{experim}}(t)$ is then given by the sum of the lipid response convoluted with the instrumental response function and the pure water response $P_{H_2O}(t)$ given in Eq. (2).

$$P_{\text{experim}}(t) = \int_{\tau=0}^{t} P_{\text{lipid}}(\tau) R_{\text{inst}}(t-\tau)\mathrm{d}\tau + P_{H_2O}(t) \tag{4}$$

The convolution integral can be evaluated, if the mono-exponential decay of P_{lipid} (Eq. (3)) is introduced.

$$\begin{aligned}&\int_{\tau=0}^{t} \exp(-k_{\text{lip}}\tau) R_{\text{inst}}(t-\tau)\mathrm{d}\tau \\ &\quad = P^0_{\text{lipid}} \frac{k_1 k_2}{k_2 - k_1}\left[\frac{1}{k_1 - k_{\text{lipid}}}\left\{\exp\left(k_{\text{lipid}} t\right) - \exp(-k_1 t)\right\}\right. \\ &\qquad \left. - \frac{1}{k_2 - k_{\text{lipid}}}\left\{\exp\left(k_{\text{lipid}} t\right) - \exp(-k_2 t)\right\}\right]\end{aligned} \tag{5}$$

A derivation of Eq. (4) is provided in Appendix A.

The two rate constants that characterize the response of the DSC instrument following pressure jumps with amplitudes between 0.5 and 3.0 bar are $k = 0.22\ \text{s}^{-1}$ and $k = 0.11\ \text{s}^{-1}$. The calorimetric instrument response time provided by the suppliers is $\tau = 9$ s. This value is in an excellent agreement with $\tau = 1/k_2 = 9.1$ s obtained from pressure relaxation.

Fig. 1 illustrates the deconvolution of the power signal resulting from a small pressure jump. The dots refer to experimental power–time data pairs obtained with frequency 1/s. The dash-dotted curve shows the instrument response for water filled cells. The dashed curve displays the lipid contribution. The calculated overall signal is represented by the continuous solid curve connecting the experimental points. The graph results from fitting the experimental data to Eq. (4).

An approximate calculation of relaxation times that are larger than 20 s could be made using a simple mono-exponential fit. For this we have to assume that the response time of the DSC instrument does not significantly depend on temperature and is not changed by small pressure jumps. The accuracy of the simplified calculation depends evidently on the relative magnitudes of the relaxation times of sample and instrument as can be seen in Fig. 2. The relaxation process at $t > 20$ s is dominated by the lipid relaxation, as the water signal has practically decayed by that time. To visualize the differences in the results between the two methods we simulated DSC–response curves such as those shown in Fig. 1 using Eq. (4), and determined relaxation times only from the exponentially decaying branch of the response curve. We used as input data Gaussian-distributed relaxation times to approximate the expected behaviour of lipids. Fig. 2 shows the input values of τ as solid Gaussian curve.

The relaxation times obtained from the simple exponential fit of the decaying branch of the simulated calorimetric response curve are shown as dots with error bars. It can be seen that the error introduced by this simplified analysis is minor (1 to 2 s) within the transition range. Outside the transition range the

Table 1
Thermodynamic and volumetric parameters of DPPC and DSPC MLVs in water

	DPPC This study	DPPC Literature	DSPC This study	DSPC Literature
Gel-to-liquid crystal transition				
$T_{1/2}$ [°C] (DSD)	41.36±0.05	41.4[a]	54.65±0.05	54.1[a]
$T_{1/2}$ [°C] (DSC)	41.46±0.02 (measured at 1.3 bar)	41.47±0.03[b]	54.86±0.02 (measured at 1.3 bar)	54.78±0.04[b] 54.9[c]
$(\Delta T)_{1/2}$ [°C][d]	0.16±0.02	0.22±0.02[b]	0.15±0.02	0.27±0.03[b]
ΔH [kJ/mol]	41.46±2	36–42[e]	46±2	47.05±2.4[b] 44.4[c]
$\mathrm{d}T_{1/2}/\mathrm{d}p$ [K/kbar]	24.8±0.05	24.76±0.02[f]	25.6±0.03	
$V_{T_{1/2}}$ (P_β) [mL/g]	0.961±0.002	0.960[a]	0.970±0.002	0.973[a]
$V_{T_{1/2}}$ (L_α) [mL/g]	1.002±0.002	0.999[a]	1.018±0.002	1.022[a]
ΔV [mL/g]	0.041±0.0028	0.039[a]	0.048±0.003	0.049[a] 0.0505[b]
Pretransition				
$T_{1/2}$ [°C] (DSC)	34.4	30–36	49.9	50.87±0.4[b]
ΔH [kJ/mol]	6.2	4.2–6.7[g] 3.8±0.8[b]	6.0±1.0	5.2±0.3[b]
$V_{T_{1/2}}$ (L_β) [mL/g]	0.9514±0.0005		0.954±0.001	
$V_{T_{1/2}}$ (P_β) [mL/g]	0.9536±0.0004		0.959±0.001	
ΔV [mL/g]	0.0022±0.0007		0.005±0.0014	

[a] Nagle J.F. and Wilkinson D.A. (1978) [42].
[b] Koynova R.et al. (1996) [43].
[c] Mabrey, S. and Sturtevant, J. M. (1976) [45].
[d] Full width at half maximum.
[e] Chowdhry, B.et al. (1984) [46]; Hinz, H.-J. and Sturtevant, J. M. (1972) [44]; Koynova, R. et al. (1996) [43].
[f] Ebel H. et al. (2001) [10].
[g] Ruocco M.J. and Shipley G.G. (1982) [2].

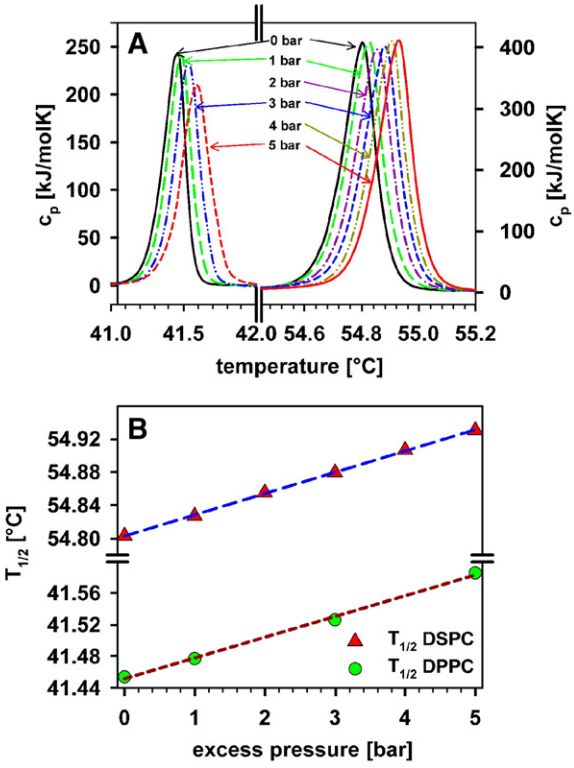

Fig. 4. A: Dependence on pressure of the main transitions of DPPC and DSPC in water. Lipid concentrations were: DPPC: 10.14 mg/mL; DSPC: 14.28 mg/mL; scan rate: 0.02 K/min. Parameters are summarized in Table 1. B: Dependence on pressure of the transition temperatures of the lipids.

relaxation time of the aqueous lipid suspension coincides practically with the relaxation time obtained with two water-filled cells, i.e. with the instrument relaxation time.

3. Results

The results are structured in the following manner. First we show DSC and DSD measurements on DPPC and DSPC vesicles in water to demonstrate consistency with published data on thermodynamic and volumetric parameters and on the dependence of the transition temperatures on pressure. Second we demonstrate the versatility of the PMDSC-method. When using slow pressure modulation formats, PMDSC permits to determine synchronously heat capacity and expansibility. Application of fast pressure changes (pressure jumps) allows the determination of relaxation times of phase transitions or conformational changes.

Fig. 3A shows heat capacity curves of multilamellar vesicles of DSPC and DPPC in water ($r = 0.2$ K/min).

The pretransitions are shown as magnified insets. The $L_\beta \rightarrow P_\beta$ and the $P_\beta \rightarrow L_\alpha$ transitions are well resolved and the sharpness of the peaks reflects the high cooperativity of the structural changes. A summary of the characteristic thermodynamic parameters is given in Table 1. The values are in good agreement with published data.

In Fig. 3B the corresponding changes in specific volume are shown as measured by differential scanning densitometry. The specific volumes change synchronously with heat capacity.

As a result of the large volume changes associated with the $L_\beta \rightarrow L_\alpha$ transitions these conformational changes are very pressure sensitive. According to the Clausius Clapeyron equation the transition temperature $T_{1/2}$ shifts with pressure.

$$\frac{dT_{1/2}}{dp} = \left(\frac{\Delta V}{\Delta S}\right)_{T_{1/2}} = T_{1/2}\left(\frac{\Delta V}{\Delta H}\right)_{T_{1/2}} \tag{6}$$

ΔH and ΔV are the molar transition enthalpy and the molar volume change, respectively, at $T_{1/2}$. The lipid transitions will occur at higher temperatures when the pressure is increased. This is demonstrated in Fig. 4A for different pressures. We employed a slow heating rate of 0.02 K/min to exclude kinetic effects on the shape of the transition curves. The slight decrease of the maximum of the heat capacity curve of DPPC concomitant with broadening is likely due to the fact that the four measurements were performed during 2 days using the same filling. It is well known that MLVs experience changes in cooperativity on repeated heating over long periods of time without significant effects on ΔH and $T_{1/2}$. The reason why DSPC did not exhibit similar changes is likely due to the shorter overall measuring time for DSPC compared to DPPC.

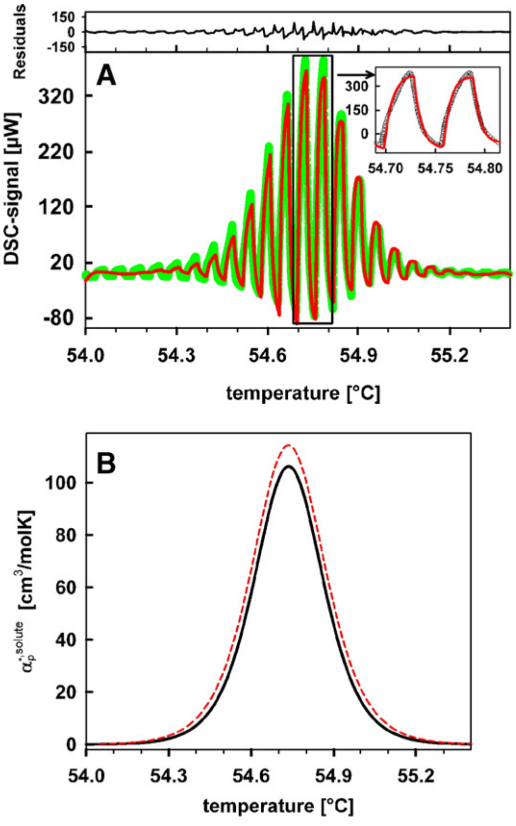

Fig. 5. Applications of pressure modulated calorimetry. A: PMDSC-measurement on DSPC (green curve) with $r = 0.02$ K/min, $\Delta p = 3$ bar and $dp/dT = 0.01$ bar/s. Red line: fit curve. Residuals are shown on top. The inset shows a magnified view of a PMDSC curve near $T_{1/2}$. B: Apparent molar expansibilities of DSPC derived from the PMDSC measurement (solid line) shown in A in comparison to data derived from DSD-measurements (dashed line). The figure has been reproduced from Ref. [6]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The variation with pressure of the transition temperatures of the $L_\beta \rightarrow L_\alpha$ transitions is plotted in Fig. 4B. The transition temperatures of both lipids depend linearly on excess pressure and the slopes of the lines are very similar. Linear fits yield 25.3 K/kbar for DPPC and 25.6 K/kbar for DSPC. We also checked the pressure coefficient using a heating rate of 0.002 K/min and observed the same value.

The knowledge of volumetric and thermodynamic parameters at one bar together with the pressure coefficient of the transition temperature provides a good basis for a new kinetic application of Pressure Modulated Differential Scanning Calorimetry (PMDSC) i.e. the determination of conformational relaxation times.

In recent publications we described theory and practice of PMDSC [6,7] for slow saw-tooth like pressure ramps or sinusoidal changes, which permit the synchronous determination of heat capacity and expansibility. The results for DSPC are shown in Fig. 5 which has been reproduced from Ref. [6].

Fig. 5A illustrates the PMDSC scan and Fig. 5B shows the transitional expansibility of DSPC. These results characterize equilibrium properties of the $L_\beta \rightarrow L_\alpha$ transition of the lipid.

Equally significant for the molecular understanding of the transitions is the time scale of conformational changes. Slow components of transitions should be measurable with a calorimeter as long as their relaxation times are larger than the instrumental time constants.

We describe in the following a method which allows determination of relaxation times of lipid phase transitions using a DSC instrument and small pressure jumps. The magnitude of the pressure jumps was 0.5 bar or 3 bar. The upper limit is dictated by the consideration not to damage the calorimeter by overpressure. The determination of relaxation times should in principle be made at constant temperature. However, as a DSC scan requires a non-zero heating rate, we approximated isothermal conditions by using very slow heating rates of 0.001 or 0.002 K/min. This guarantees that during the time required for relaxation of the pressure-induced power signal the temperature changes are sufficiently small to render them insignificant with regard to temperature-dependent changes in thermodynamic properties. In any case the temperature changes are well defined and smaller than the random temperature fluctuations reported for experiments performed with large pressure jumps (Δp between 40 and 80 bar) under isothermal conditions [9].

Fig. 6 shows a summary of the DSC signals that result from the application of small pressure jumps during a DSC scan to aqueous suspensions of MLVs of DPPC and DSPC.

The heating rate used for DSPC was 0.002 or 0.001 K/min. The pressure jumps involved pressure increases and pressure

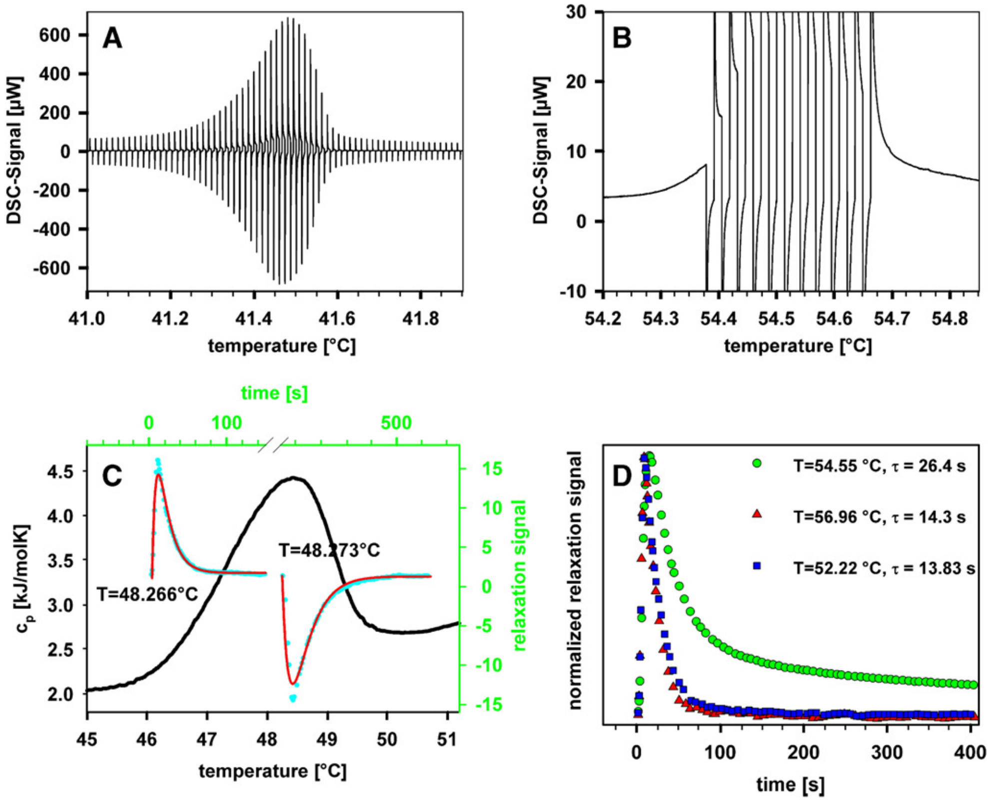

Fig. 6. Calorimetric pressure jump studies on multilamellar vesicles of DPPC and DSPC in water. A: Pressure jump DSC measurements on DPPC (12.4 mg/mL); scan rate: 0.002 K/min. B: Pressure jump DSC on DSPC (5.0 mg/mL); scan rate: 0.002 K/min; the graph shows the heat capacity curve with pressure jumps in the main transition region. C: Heat capacity curve of the pretransition of DSPC (left axis) and the signals resulting from a negative pressure jump (3.3 bar to 0.3 bar) followed after 400 s by a positive pressure jump (0.3 bar to 3.3 bar). The jumps occur close to $T_{1/2}$ of the pretransition. D: Normalized relaxation curves of DSPC at the transition temperature (green curve), before the start of the main transition (blue curve) and after the transition (red curve). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

decreases of 3 bar between 0.3 and 3.3 bar. The period between jumps was 160 sec or 400 s. Depending on the direction of the pressure change one observes negative or positive power peaks. With decreasing pressure the transition temperature shifts downwards. Such a pressure jump will therefore be associated with additional melting of lipids, which in turn requires heat input from the calorimeter to keep ΔT between the cells zero. Since the heating system responds practically instantaneously, the recorded power signal resulting from the negative pressure jump is a convolution of the instrument response and the characteristic uptake of heat by the lipid vesicle from water.

In analogy to Grabitz et al. [9] we assumed heat transfer from and to the lipid to be single exponential. The resulting function that describes then quantitatively the relaxation behaviour of the lipids is Eq. (4). Positive pressure jumps involve the opposite phase change. Melting is reversed with concomitant release of heat from the lipid vesicles into water. As the DSC instrument cannot actively cool during a heating scan, heat dissipation can, at least in principle, not assumed to be instantaneous. This renders the situation different from the instantaneous activation of heating in case of negative pressure jumps. It can, however, be assumed that, for very small positive pressure jumps such as 0.5 bar, heat dissipation is fast enough so that the resulting relaxation curves can also be evaluated with but small errors.

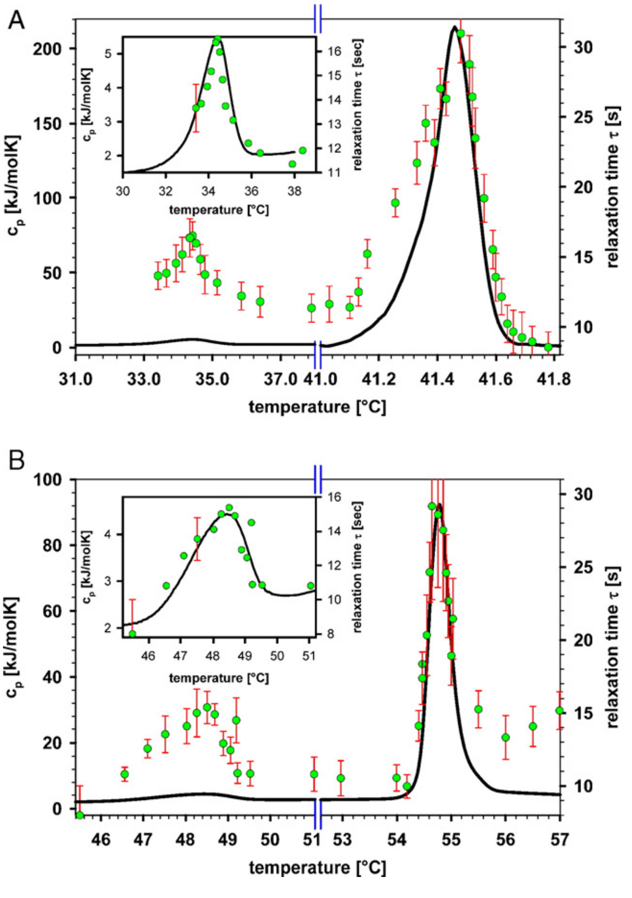

Fig. 7. Relaxation times and heat capacity scans of DPPC and DSPC multilamellar suspensions in water. A: DPPC (12.4 mg/mL); B: DSPC (5.0 mg/mL). The insets show the heat capacity curves of the pretransition together with the corresponding relaxation times. The highest relaxation times are observed at the midpoint of the transitions. All relaxation times scale with heat capacity.

The relaxation times we obtained for aqueous suspensions of DPPC and DSPC together with the corresponding heat capacity curves are illustrated in Fig. 7.

We included in our analysis DPPC for comparison. The relaxation behaviour of its main transition had been characterized before in ref. [9], however, by a somewhat different method. These authors had applied large pressure jumps–up to 80 bar–to a lipid suspension of approximately 70 mg/mL. The suspension was included in a capillary and the capillary was inserted into the sample cell of an isothermal microcalorimeter. The most important findings of that study were the following: Transitional relaxation times were in the range from about 5 to 40 s and they scaled with heat capacity. The highest value was observed at the midpoint temperature $T_{1/2}$ of the transition.

The present results support the previous findings for the main transition of DPPC. The relaxation times change in proportion to c_p and their absolute values are within error limits identical in both the present study and ref. [9].

We were able to determine also relaxation times for the pretransition, which had not been reported previously. It can be seen in Fig. 7A that the maximal τ value characteristic for the pretransition is approximately half as large (17 s) as that for the main transition. The relaxation times follow, however, also the shape of the pretransitional heat capacity peak.

Fig. 7B exhibits the first results on relaxation behaviour of DSPC. It is evident that the relaxation times of the main transition mimic again the temperature course of heat capacity. The magnitude of τ values varies between 9 s and 30 s at $T_{1/2}$. The relaxation times of the pretransition vary also in proportion to heat capacity as the graph illustrates. Surprisingly the absolute values of the relaxation times of both lipids are identical within error limits.

4. Discussion

Relaxation phenomena in multilamellar lipid vesicles have been studied previously by laser T-jump real time X-ray measurements [5,17–28], by pressure jump techniques [3,4] as well as by ultrasonic-, dielectric, and T-jump relaxation techniques [29–38].

Two or three relaxation times emerged as general pattern in most studies that were practically independent of the lipid system studied. Depending on the method of detection relaxation times were observed in different time windows. Processes in the nanosecond time regime were detected by ultrasound absorption [33–35,38–40]. They were identified as cooperative phenomena on the basis of the observation that relaxation times exhibited a maximum at the midpoint of the transition. Relaxation times between 0.1 ms and 470 ms were measured on phosphatidic acid bilayers [3] using pressure jump techniques in combination with light scattering detection. Characteristic kinetic parameters of phase transitions of various glycero- glycolipids with monosaccharide- or disaccharide head groups were determined by us using real time laser T-jump X-ray diffraction. Two or three relaxation times could be separated in the time range between about 10 and 400 ms [10,17,18,41]. The common feature of all these kinetic studies on lipid transitions was that none of the relaxation times observed by the various techniques was greater than about 1 s.

The first report of significantly larger relaxation times was that by Grabitz et al. [9]. In that study pressure jumps of 40 to 80 bar had been applied to 100 mM lipid suspensions of DMPC or DPPC. The lipid suspensions were located in a capillary immersed in the sample cell of an isothermal calorimeter. The pressure-induced phase changes of the phospholipids were recorded as time-dependent power pulses. Two particularly intriguing results of those studies pertinent to our findings are noticeable. First, the maximal relaxation times at the respective midpoints of the transitions of DMPC and DPPC were approximately 35±5 s for both lipids. Second, the relaxation times changed in proportion to transitional heat capacity. We obtained practically identical results for DPPC, although our experimental approach was significantly different from that of ref. [9]. We employed much smaller pressure changes, 0.5 or 3 bar instead of 40 to 80 bar. Furthermore the pressure jumps were applied directly to the calorimetric cells during a slow heating scan ($r=0.001$ or 0.002 K/min) in a continuous manner: positive jump-waiting period–negative jump. Therefore the temperature was always precisely defined and changed within 3 relaxation times, i. e. within approximately 100 s, by less than 0.002 K, if a heating rate of 0.001 K/min was used. Thus, strictly speaking, we did not establish isothermal conditions. However, these controlled changes of temperature are much smaller than the random temperature fluctuations resulting from pressure jumps of 40 or 80 bar in an isothermal calorimeter. Therefore we are confident that the minute non-isothermicity does not affect the relaxation times in any significant manner. The second aspect by which our experimental set up differs from that of [6] is that the lipid vesicles are directly in the calorimetric cell and not in a separate capillary and that their concentrations are by a factor of 5 lower. We could have studied even lower concentration of about 1 mg/mL, if we had restricted the measurements to the main transition. However, as we wanted to determine also relaxation times of the pretransition, power signals would have become undetectable at such low concentrations in combination with the low heating rates.

In view of these experimental differences it is the more surprising that our result on DPPC ($\tau=30\pm4$ s) is in excellent agreement with that of ref. [9]. It was, however, unexpected that the relaxation time obtained for the C-18 phospholipid DSPC is also practically identical to those observed for DMPC and DPPC. This finding suggests a somewhat different interpretation of the molecular origin of the pressure-induced relaxation phenomenon of phospholipids than given in ref [9]. It had been proposed there that the main relaxation process is not an activated process with an activation barrier, but rather the result of the large degeneracy of states with similar free energy in the vicinity of the heat capacity maximum The relaxation time is dominated by the mean time which the system needs to undergo a random walk through all the degenerate states surrounding the equilibrium states. At the heat capacity maximum the degeneracy of states with about equal free energy is maximal, so is therefore the relaxation time.

We can agree with the analysis with regard to the proportionality between heat capacity and transitional relaxation times. However, in view of the differences in the heat capacity maxima of DPPC and DSPC seen in Figs. 3 and 7 one would expect to find also differences in the relaxation times of the lipids, if the relationship $\tau=(T^2)/L\equiv\alpha\, c_\mathrm{p}$ between relaxation time τ and heat capacity derived in [9] was strictly valid. In the formula T is absolute temperature, L is a phenomenological coefficient that provides the connection between thermodynamic fluxes and thermodynamic forces, c_p is heat capacity, and α is the proportionality constant between relaxation time and heat capacity.

In view of the differences in the heat capacity maxima and the transition temperatures of the lipids and the similarities of the corresponding relaxation times, the molecular origin of the relaxation phenomena for DMPC, DPPC, and DSPC needs another explanation. It appears reasonable to assume that the same molecular process should be responsible for the occurrence of nearly identical τ values. Recently Rappolt et al. [28] reported IR-laser-induced time-resolved X-ray diffraction studies on DPPC in which they observed an intermediate anomalous thin lamellar structure when increasing temperature through the pretransition. A similar non-equilibrium structure was observed by Pabst et al. [19] when performing T-jump studies on the L_α-phase of POPC to characterize elastic properties of bilayers. In both cases intermediate bilayer expansion and compression normal to the bilayer surface was involved in the structural change. The relaxation of the d-spacings of the intermediate structure could be fitted to a double exponential decay model yielding a fast constant of 0.45 s and a slower component of 3 s. However, most importantly they also observed a very slow relaxation process of about 30–40 s associated with the recovery of both the Bragg peak intensity and the full width at half height (fwhm). The authors assigned this relaxation tentatively to diffusion of water and passive cooling, without being able to separate these two phenomena. Water was assumed to diffuse back into the interbilayer water region from the bulk to regenerate the equilibrium structure from the anomalously compressed form.

We assume that we observe a similar process in the pressure-induced DSC relaxation studies. We do not consider the agreement between the relaxation times determined in the present measurements and those observed in the T-jump X-ray diffraction studies accidental. The only parameter which pressure acts upon is the difference in volume between the various phases of the multibilayers. Positive and negative pulses of external pressure are therefore likely to cause water fluxes in the interbilayer region that relax with the observed long time constants. As DMPC, DPPC, and DSPC have the same head group structure, interbilayer hydration can be assumed to be similar. This would be a reasonable explanation for the similarity of the relaxation times observed for the pressure-induced water fluxes despite the differences in their hydrophobic alkyl chain region. The fast changes identified for the *trans*-gauche-isomerisations of the alkyl chains are therefore not relevant to the pressure-induced enthalpy fluxes seen in the calorimeter.

Inspection of the relaxation times occurring between the pretransition and the main transition reveals an interesting phenomenon. As can also be seen in heat capacity curves, after the pretransition the system is in a state different from that before the transition. Relaxation times are higher than those before the pretransition as well as those after the main transition. In line with the hydration interpretation given for the relaxation phenomena characteristic of the main transition we propose analogous phenomena to occur at a reduced scale on changing from L_β to P_β. These interpretations appear to be reasonable in view of the significant equilibrium volume changes associated with the two transitions as shown in Fig. 3B.

Appendix A

Under the assumption that a) heat release or absorption caused by a pressure jump in water filled calorimetric cells can be described with a Dirac delta function, δ, and b) that this heat is transferred to the cell wall with a rate constant k_1 and then released from the cell wall with a rate constant k_2 the following convolutions will provide the power response signal of the calorimeter. The first normalized folding (rate constant k_1 as normalizing factor) results in an exponential function:

$$\int_{-\infty}^{t} \delta(\tau)k_1 h(1-\tau)\mathrm{d}\hat{o} = k_1\exp(-k_1 t)$$

The second folding using $h(t)=k_2 \exp[-k_2 t]$ results in the equation

$$\begin{aligned}
&\int_0^t k_1\exp(-k_1\hat{o})k_2 h(t-\hat{o})\mathrm{d}\hat{o}\\
&\quad = k_1k_2\int_0^t \exp(-k_1\hat{o})\exp[-k_2(t-\tau)]\mathrm{d}\hat{o}\\
&\quad = k_1k_2\exp(-k_2t)\int_0^t \exp((k_2-k_1)\tau)\mathrm{d}\hat{o}\\
&\quad = k_1k_2\exp(-k_2t)\frac{1}{k_2-k_1}\left[\exp((k_2-k_1)\tau)\right]_0^t\\
&\quad = k_1k_2\exp(-k_2t)\frac{1}{k_2-k_1}\left[\exp\{(k_2-k_1)t\}-1\right]\\
&\quad = \frac{k_1k_2}{k_2-k_1}(\exp(-k_1t)-\exp(-k_2t))
\end{aligned}$$

This normalized function can be considered the instrument response function.

$$R_{\mathrm{inst}}(t) = \frac{k_1k_2}{k_2-k_1}(\exp(-k_1t)-\exp(-k_2t))$$

If the calorimetric cells contain lipids in addition to water, the heat flow scheme resulting from pressure jumps will generally be different. In the simplest case we can take this into account by assuming a mono-exponential heat release from the lipid vesicles with a rate constant k_{lipid}. The corresponding heat flow scheme is then:

$$\text{lipid} \xrightarrow{k_{\mathrm{lipid}}} \text{water} \xrightarrow{k_1} \text{cell wall} \xrightarrow{k_2} \text{detector}$$

The proper response function for this heat transfer sequence is Eq. (4).

To obtain Eq. (4), the assumed mono-exponential heat release function, P_{lipid}, of the lipid vesicles has to be folded with the normalized instrument response function, R_{Instr}.

$$P_{\mathrm{lipid}}(t) = P^0_{\mathrm{lipid}}\exp(-k_{\mathrm{lipid}}t)$$

$$R_{\mathrm{Inst.}}(t) = \frac{k_1k_2}{k_2-k_1}(\exp(-k_1t)-\exp(-k_2t))$$

$$\begin{aligned}
P_{\mathrm{experim.}} &= \int_0^t P^0_{\mathrm{lipid}}\exp\left(-k_{\mathrm{lipid}}\tau\right)\frac{k_1k_2}{k_2-k_1}\Big[\exp\{-k_1(t-\tau)\}\\
&\quad -\exp\{-k_2(t-\tau)\}\Big]\mathrm{d}\tau + P_{\mathrm{H_2O}}\\
&= P^0_{\mathrm{lipid}}\frac{k_1k_2}{k_2-k_1}\int_0^t \exp\left(-k_{\mathrm{lipid}}\tau-k_1t+k_1\tau\right)\\
&\quad -\exp\left(-k_{\mathrm{lipid}}\hat{o}-k_2t+k_2\tau\right)\mathrm{d}\tau + P_{\mathrm{H_2O}}\\
&= P^0_{\mathrm{lipid}}\frac{k_1k_2}{k_2-k_1}\int_0^t \exp\left[\left(k_1-k_{\mathrm{lipid}}\right)\tau-k_1t\right]\\
&\quad -\exp\left[\left(k_2-k_{\mathrm{lipid}}\right)\tau-k_2t\right]\mathrm{d}\tau + P_{\mathrm{H_2O}}\\
&= P^0_{\mathrm{lipid}}\frac{k_1k_2}{k_2-k_1}\Big[\exp(-k_1t)\int_0^t \exp\{(k_1-k_{\mathrm{lipid}})\tau\}\mathrm{d}\tau\\
&\quad -\exp(-k_2t)\int_0^t \exp\{(k_2-k_{\mathrm{lipid}})\tau\}\mathrm{d}\tau\Big] + P_{\mathrm{H_2O}}\\
&= P^0_{\mathrm{lipid}}\frac{k_1k_2}{k_2-k_1}\left[\frac{\exp(-k_1t)}{k_1-k_{\mathrm{lipid}}}\left(\exp\left\{\left(k_1-k_{\mathrm{lipid}}\right)t\right\}-1\right)\right.\\
&\quad \left.-\frac{\exp(-k_2t)}{k_2-k_{\mathrm{lipid}}}\left(\exp\left\{\left(k_2-k_{\mathrm{lipid}}\right)t\right\}-1\right)\right] + P_{\mathrm{H_2O}}\\
&= P^0_{\mathrm{lipid}}\frac{k_1k_2}{k_2-k_1}\left[\frac{1}{k_1-k_{\mathrm{lipid}}}\left\{\exp\left(k_{\mathrm{lipid}}t\right)-\exp(-k_1t)\right\}\right.\\
&\quad \left.-\frac{1}{k_2-k_{\mathrm{lipid}}}\left\{\exp\left(k_{\mathrm{lipid}}t\right)-\exp(-k_2t)\right\}\right] + P_{\mathrm{H_2O}}
\end{aligned}$$

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