# A Novel Method To Evaluate the Phase Transition Thermodynamics of Langmuir Monolayers. Application to DPPG Monolayers Affected by Subphase Composition

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There is lack of thermodynamic studies to describe the LE-LC 2D-phase transition of dipalmitoyl phosphatidyl glycerol (DPPG) although it is one of the main components of lung surfactants. Spread monolayers of synthetic DPPG are investigated in a broad temperature range on subphases modeling the native biological substrate. The phase transition pressure changes linearly with temperature, which confirms the principal applicability of the two-dimensional Clausius−Clapeyron equation to evaluate thermodynamic data of such systems. Brewster angle microscopy performed at monolayer compression shows that it is strongly heterogeneous during phase transition. The competition between strong condensation effects of $Ca^{2+}$ cations on the negatively charged DPPG anions and electrostatic repulsion due to $Na^{+}$ ions preventing condensation leads to a very fast growth of the LC phase. As a consequence, strong irregularities in the domain shapes and size distribution are observed. A new approach is proposed to determine the molar area at the phase transition from the set of $\pi/A$ isotherms recorded at different temperatures. The two-dimensional Maxwell relation and Clausius−Clapeyron equation were simultaneously applied to calculate the required parameters with a reasonable accuracy. The entropy changes at the phase transition, transition heats, and the critical temperature are calculated.

## Introduction

The continuously increasing number of publications devoted to lung surfactants and to their individual components as separate substances reflects the great importance of such systems especially in fields of human physiology[1,2] and biophysical chemistry.[3−5] Because the physiological relevance of such objects is determined by the unique properties of the system as a whole, it is very difficult to investigate strictly their physicochemical properties. Indeed, the chemical composition of lung surfactants is extremely complicated as the number of main components even in simplified systems exceeds two. Additionally, the corresponding underlying or surrounding bulk phase in lungs is also a multicomponent system. This is the reason the majority of thermodynamic investigations have been carried out for relatively simple systems containing either one or few main components of lung surfactants.[6−8] However, whereas model systems containing only dipalmitoyl phosphatidyl choline (DPPC) were investigated very extensively in the last 2 decades,[6,9−14] especially in form of spread monolayers, the other main lipid component, dipalmitoyl phosphatidyl glycerol (DPPG), was characterized quite rarely from a thermodynamic point of view.[8,15−17] Moreover, the published data do not contain some of the basic thermodynamic characteristics of DPPG monolayers, and several measured parameters are in contradiction to each other.[8,15,16]

Therefore, this paper aims to close partially the existing deficiency and to clarify the origin of some disagreements found in the literature. A pure DPPG monolayer was investigated in order to simplify the chemical composition of the monolayer and, consequently, the respective thermodynamic description. The composition of the subphase and the conditions under which the system was investigated were selected from a physiological point of view.

First, a buffer solution with additions of $Na^{+}$ and $Ca^{2+}$ ions was taken as the subphase imitating the chemical composition of physiological fluids. Second, the temperature range was chosen between 298 and 314 K to mimic the condition in the living systems. More details to these procedures are given below in the experimental part.

The main information on the physicochemical behavior of monolayers was obtained from conventional $\pi/A$ isotherms measured at different temperatures. In addition, a simultaneous recording of the monolayer structure by Brewster angle microscopy (BAM) was instructive for a qualitative characterization of the monolayer state at different stages during compression.

## Experimental Section

Measurements of the $\pi/A$ isotherms were carried out on a rectangular Langmuir trough made from PTFE with a total surface area of about 518 $cm^2$. A microbalance with a Wilhelmy plate was used to monitor the surface pressure changes upon monolayer compression, which was performed asymmetrically with a moving barrier at constant area velocity of 0.25 $cm^2/s$. The constant spread volume of a stock solution of DPPG ($10^{-3}$ M in 1/3 methanol/chloroform, v/v) leads to a constant compression rate of about $6.97 \times 10^{-4}$ $nm^2$ $molecule^{-1}$ $s^{-1}$. Such experimental conditions allow keeping slow relative

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compression rates even in the plateau region of the $\pi/A$ isotherm. The monolayers were spread in a conventional way by means of a Hamilton microsyringe. The spread amount of the stock solution was chosen in order to reach a large initial molecular area corresponding to the liquid-expanded state of the monolayer. The recording of $\pi/A$ isotherms was started about 10 min after spreading so that the monolayer became equilibrated during this time. Each measurement of an isotherm was repeated several times (at least three times) until a satisfactory reproducibility of the data was reached.

Attention was paid to the preparation of the subphase on which the DPPG monolayers were spread. TRIZMA 0.05 M buffer solution was chosen as substrate modeling the pH values in biological fluids. The composition containing 7.02 g of TRIZMA HCl and 0.67 g of TRIZMA Base dissolved in 1 L of water allows solutions with pH = 6.91 at 310 K. At lower temperatures the pH values are slightly shifted toward basic solutions up to pH = 7.20 at 298 K. The ionic strength of the subphase was determined by addition of NaCl at a concentration of 150 mM. Another important part of the composition of biological fluids that can strongly affect the properties of lung surfactants is a small admixture of $Ca^{2+}$ ions. Therefore, 2 mM $CaCl_2$ was added to the subphase for imitating of influence of $Ca^{2+}$ ions on the spread DPPG layers. The purity of the described subphase was controlled in a conventional way by simultaneous compression of the bare substrate and BAM studies. In all cases the appropriate grade of purity was reached after several repeated cleaning procedures consisting in a fast movement of the barrier over the trough surface with subsequent sucking off of the surface-active impurities.

The Langmuir trough was embedded into the BAM device[16] so that the measurement of $\pi/A$ isotherms could be carried out simultaneously with the video recording.

The evaporation of the subphase during the experiment caused by the very large surface of the Langmuir trough can affect the force detector data and the composition of the subphase especially at temperatures essentially higher than room temperature. For that reason the humidity of the air around the whole experimental setup was saturated by water vapor to avoid possible artifacts.

DPPG was purchased from Sigma in form of the sodium salt and used without any additional processing. Methanol (spectroscopic grade from Sigma) and chloroform (Fluka) were used as spreading solvent without extra purification. Sodium chloride (Riedel-de-Haen, p.a. purification grade) was heated at 600 °C before use to remove possible surface-active impurities of organic origin. The TRIZMA Base (purification grade >99.9%) and TRIZMA HCl salts (purification grade >99%) used for the preparation of the TRIZMA buffer solution were purchased from Sigma. The $CaCl_2 \cdot 2H_2O$ from Fluka (for molecular biology, 99.5%) was taken as a source of $Ca^{2+}$ ions. Milli-Q deionized water with a specific electric conductivity of 18.2 MΩ·cm was used to prepare the subphase.

## Results and Discussion

The $\pi/A$ isotherms for the spread DPPG layers at temperatures between 298 and 314 K are shown in Figure 1, where some qualitative peculiarities can be observed. At first, the isotherms are not completely regular shifted with increasing temperature toward higher pressures. For example, the isotherms at 298 and 300 K differ from each other only by approximately 1 mN/m whereas the isotherms at 303 and 305 K exhibit a surface pressure changes of about 3−4 mN/m. At higher temperatures the isotherm shift decreases again down to 2 mN/m. Such

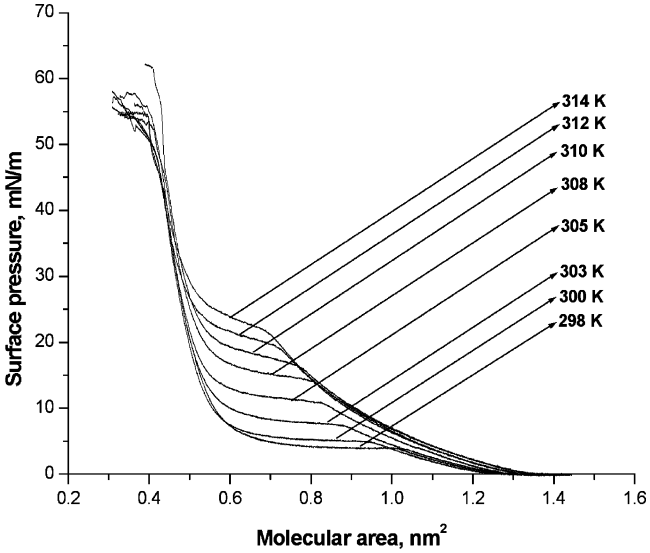


**Figure 1.** Pressure/area isotherms for DPPG monolayers in the temperature range between 298 and 314 K.

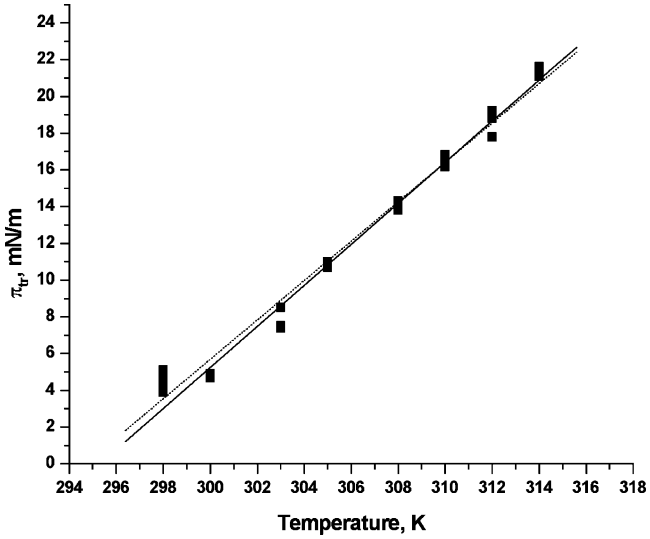


**Figure 2.** Transition pressure for DPPG monolayers as a function of temperature: dotted line, best linear fit for all experimental points; solid line, best linear fit without three most deviating points at 298 K.

irregularities have been already observed by other authors, for example, by Moore at al.[18] or Albrecht et al.[6] In contrast, the DPPG isotherms on pure water reported recently by Vollhardt et al.[8] change in shape quite regularly with increasing temperature. The origin of such behavior can be the influence of the temperature on the size and shape of aggregates,[19] leading to corresponding small changes in the shape of $\pi/A$ isotherms or of the sensitivity to traces of impurities.

Nevertheless, the temperature dependence of the surface pressure at the LE−LC phase transition kink point in the $\pi/A$ isotherm (transition pressure $\pi_{tr}$) is quite linear (Figure 2), although some experimental points are quite far from the rest and could be considered as erroneous. To exclude erroneous points, the following criterion was used. The original set of experimental data was fitted to a line (dashed line in Figure 2) and the overall standard deviation $\sigma_f$ was calculated at a confidence level of 0.95. Then, all points outside the interval $\pm 2\sigma_f$ were considered as erroneous. The second fit in Figure 2 (solid line) corresponds to the improved set after excluding all erroneous points. The described procedure leads to an improved correlation coefficient $R$ for $\pi_{tr}(T)$ from 0.992 to 0.995 and of

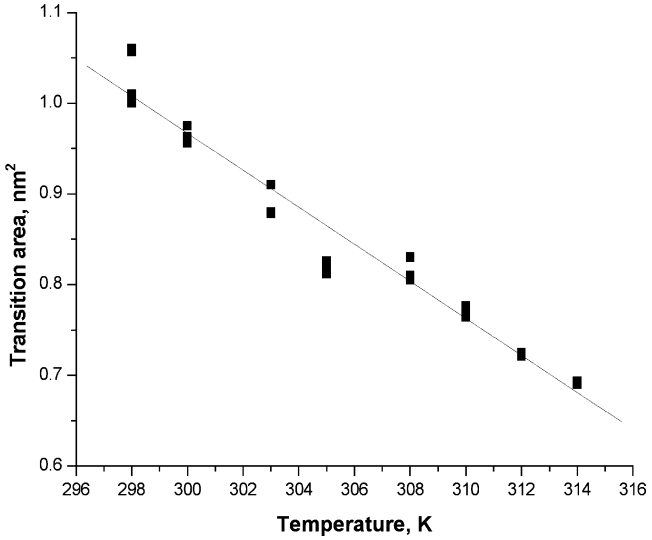


**Figure 3.** Temperature dependence of the transition area for DPPG monolayers: solid line, best linear fit.

the standard deviation from 0.77 to 0.60. Figure 3 represents the temperature dependence of the transition area $A_{tr}$, which corresponds to the transition pressure. The transition areas decrease, in contrast to the transition pressure, almost linearly with increasing temperature; however, the deviation from a linear fit is more remarkable due to the relatively large spreading error. On the other hand, the linear dependence even at highest temperatures indicates no significant loss of monolayer substance due to increasing solubility.

A quantitative thermodynamic analysis of the main two-dimensional phase transition in the monolayer can be based on different approaches. For example, Gershfeld and Pagano[20] have used a numerical integration of the experimental $\pi/A$ isotherm to find changes in the Helmholtz free energy during film compression $\Delta F_c$. Another approach is connected with the two-dimensional Clausius−Clapeyron equation

$$\frac{d\pi_{tr}}{dT} = \frac{S_{LE} - S_{LC}}{A_{LE} - A_{LC}} \tag{1}$$

where $S_{LE}$, $A_{LE}$, $S_{LC}$, and $A_{LC}$ are the molecular entropy and area in the liquid-expanded and liquid-condensed states, respectively. The change of entropy during the phase transition can be evaluated from eq 1, and the corresponding transition heat $\Delta Q_{tr}$ can be estimated by multiplying with the phase transition temperature. The main problem with both approaches is the exact determination of $A_{LC}$.[21] In the first approach, this quantity is needed as the upper limit of integration, and eq 1 requires also knowledge of this parameter. The problem can be avoided if $A_{LC}$ is determined independently. One of such possibilities is the application of the grazing incidence X-ray scattering (GIXS)[22] method simultaneously with the conventional $\pi/A$ isotherm measurements. The quantitative evaluation of BAM pictures allows also a quite accurate and independent determination of $A_{LC}$.[16] However, it leads to sufficiently accurate values of $A_{LC}$ only if the size and two-dimensional density of domains in the plateau region are homogeneous over the whole monolayer area.[21]

Another approach for the independent evaluation of the phase transition thermodynamics in monolayers, i.e., determination of $A_{LC}$, is the application of the Butler equation

$$\mu_i = \mu_i^0 + RT \ln f_i x_i - \gamma \omega_i \tag{2}$$

to both coexisting 2D phases in equilibrium.[8,23] Here $\mu_i^0$ is the standard chemical potential of component $i$ in the surface layer depending only on temperature and pressure, $f_i$ is its surface activity coefficient, $x_i$ is its mole fraction in the surface layer, $\gamma$ is the surface tension, and $\omega_i$ is the partial molar area of the component $i$ in the surface layer. The standard free energy of the two-dimensional phase transition can then be expressed as[8]

$$\Delta G^\circ = RT \ln(\{\omega_{(n)}\}/\{A_{tr}\}) \tag{3}$$

where $\omega_{(n)}$ is the molar area of surface-active component in the aggregated state.

In the present experiments the DPPG monolayers were spread on a physiological subphase so that a direct comparison with results given in ref 8 is not possible. The chemical composition of such a subphase containing $Na^+$ and $Ca^{2+}$ ions has a strong influence on the properties of the monolayer.[24,25]

The new thermodynamic approach proposed here is based on the evaluation of the whole set of the $\pi/A$ isotherms measured at different temperatures and a subsequent application of the two-dimensional Maxwell relation and Clausius−Clapeyron equation. The basics of this approach can be formulated as follows. The entropy changes at constant temperature can be derived from Maxwell's relation in the following form

$$\frac{dS_T}{d\pi} = -\left(\frac{\partial A}{\partial T}\right)_\pi \tag{4}$$

Hence, the entropy change between two monolayer states corresponding to certain surface pressures $\pi_1$ and $\pi_2$ can be expressed as

$$\Delta S_T = -\int_{\pi_1}^{\pi_2} \left(\frac{\partial A}{\partial T}\right)_\pi d\pi \tag{5}$$

where the subscript T denotes the constancy of temperature. The right-hand side of eq 5 can be integrated numerically. To do so, the partial derivative of the molecular area with respect to temperature at constant surface pressure must be presented as a function of surface pressure. The integration limits $\pi_1$ and $\pi_2$ are the surface pressures between which the phase transition has to be estimated. Any pressure value just after the beginning of the phase transition can be easily taken as $\pi_1$. The second pressure value $\pi_2$ can be chosen on the part of a $\pi/A$ isotherm after the plateau region. Note, that in the present case the chosen position of $\pi_2$ on the $\pi/A$ isotherm is less critical as in other thermodynamic approaches discussed above and does not lead to large relative errors in $A_{LC}$. The function $(\partial A/\partial T)_\pi = f(\pi)$ in the integration limits can be obtained from the experimental $\pi/A$ isotherms at a given temperature.

A numerical evaluation of the dependencies $A = f(T)$ is the first stage of the calculation. The experimental $\pi/A$ isotherms were intersected by horizontal lines representing the different constant surface pressures taken between $\pi_1$ and $\pi_2$. Figure 5 shows this calculation procedure schematically for the $\pi/A$ isotherm measured at the highest temperature (314 K), because the application of this isotherm yields the dependence $A = f(T)$ with a maximum number of intersection points. The values of $\pi_1$ and $\pi_2$ used for the calculation were 21.6 and 31.6 mN/m, respectively, and the pressure step was 1 mN/m. The dependence $A = f(T)$ determined at $\pi = 22.6$ mN/m is shown as an example in the insert of Figure 5.

At the second stage of the calculation procedure, the dependencies $A = f(T)$ are fitted by a polynomial and the derivatives $(\partial A/\partial T)_\pi$ calculated (see solid line with squares in Figure 6). The point of lowest-pressure deviates strongly from

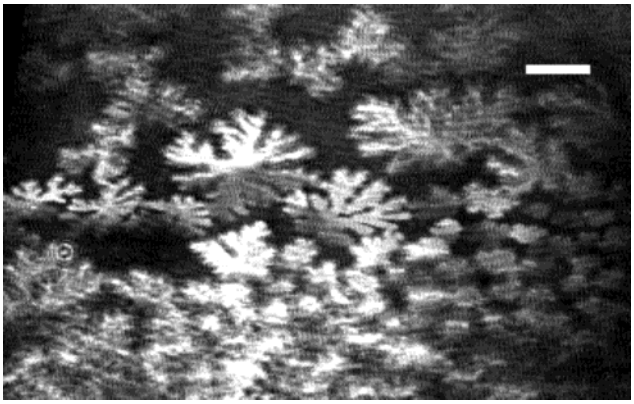

**Figure 4.** Strongly branched shapes of growing domains of the LC phase and inhomogeneities in the matter distribution within the DPPG monolayer in the plateau region: white bar, 50 $\mu$m; the image was taken at $T = 300$ K and $A = 0.65$ nm$^2$/molecule.

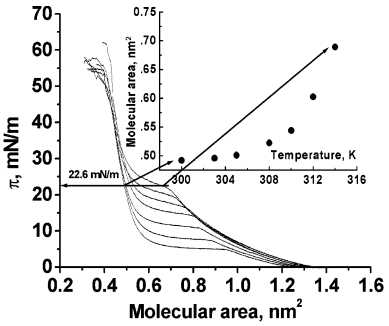


**Figure 5.** Schematic demonstration of the procedure to determine the molecular area at different temperatures and constant pressure ($\pi =$ 22.6 mN/m).

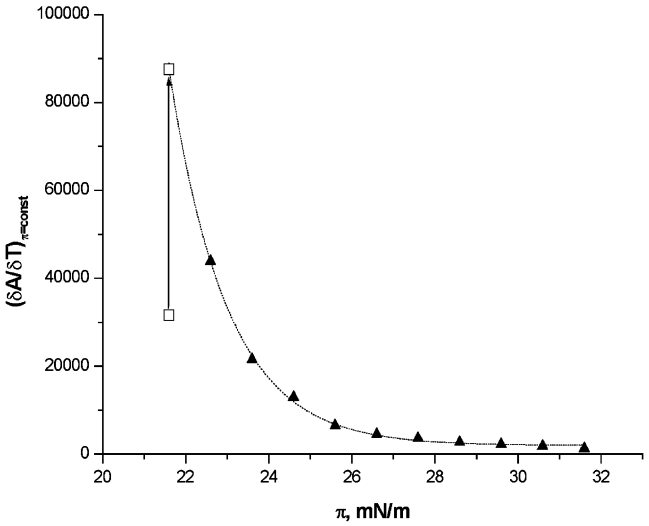


**Figure 6.** Dependence of $(\partial A/\partial T)|_{\pi=\text{const},T=314\text{K}}$ on surface pressure $\pi$ (filled triangles): dotted line, best fit (exponential decay) taking into consideration only the points in the pressure range between 22.6 and 31.6mN/m; bottom open square, initial value of $(\partial A/\partial T)|_{\pi=\text{const},T=314\text{K}}$ at $\pi = 21.6$ mN/m; top open square denotes the improved value of the derivative (for more details see text).

other points. The most probable explanation could be seen in the quality of the isotherm, measured at 314 K, and the proximity of $\pi_1$ and the corresponding $A$, to the point of the phase transition onset ($\pi_{tr}$; $A_{tr}$). The possible weak supersaturation or sufficient inhomogeneity in the monolayer just after the beginning of the phase transition contributes to the enhanced uncertainty of this point. Moreover, even a small spreading error or a small loss of lipid due to enhanced solubility at $T = 314$ K can lead to an additional deviation in the dependence $A = f(T)$ and, consequently, to the strong deviation of the derivative $(\partial A/\partial T)_\pi$. A better value of the derivative for $\pi = 21.6$ mN/m can be obtained by fitting this dependence for 22.6−31.6 mN/m and extrapolation to $\pi = 21.6$ mN/m, which yields 87500 m$^2$/mol·K. This value is used in further calculations. For the nonlinear least-squares fitting, the Levenberg−Marquardt algorithm was applied. Figure 6 demonstrates the good agreement with the experimental data.

The entropy change during isothermal compression at $T =$ 314 K between $\pi_1 = 21.6$ mN/m and $\pi_2 = 31.6$ mN/m amounts to $\Delta S_{tr} = S_{LC} - S_{LE} = -143$ J/mol·K, which is confirmed by the two-dimensional Clausius−Clapeyron equation (1). The molecular area corresponding to $\pi_1$ in eq 5 was inserted into eq 1 as the value for $A_{LE}$. Such substitution is physically reasonable because $\pi_1$ is very close to the kink pressure $\pi_{tr}$ (see Figure 1), although some remaining difference between these values can cause significant deviation for the lowest-pressure point as was mentioned above. The value of $A_{tr}$ in turn is practically equal to $A_{LE}$ and can be replaced by it. The value on the left-hand side of eq 1 was taken from a linear fit presented by the solid line in Figure 2. According to this procedure, the value of the derivative $(\partial\pi_{tr}/\partial T)$ is 0.00118 N/m·K. The substitution of both parameters, $A_{LE}$, and $(\partial\pi_{tr}/\partial T)$ into eq 1 together with $\Delta S_{tr}$ given above allows us to obtain the value of $A_{LC}$ of 0.488 nm$^2$, in agreement with the value of the molecular area $A_2$ corresponding to $\pi_2$ which amounts to 0.484 nm$^2$. Indeed, $\pi_2$, which plays the role of the upper integration limit in the calculation of $\Delta S_{tr}$ from eq 5, was chosen arbitrarily. Therefore, the corresponding molecular area $A_2$ can be taken as $A_{LC}$ only with a certain vagueness. If another value of $\pi_2$ is chosen, the agreement between $A_{LC}$ and $A_2$ can be even improved. As the surface pressure taken as $\pi_2$ can be chosen with high accuracy, the adjustment between $A_{LC}$ and $A_2$ can be repeated as long as the values are identical within the error limit. Best agreement was found at $\pi_2 = 30.6$ mN/m: $A_{LC} = 0.490$ nm$^2$ and $A_2 = 0.491$ nm$^2$, respectively. Further reduction of $\pi_2$ increases the difference between $A_{LC}$ and $A_2$. For example, for $\pi_2 = 25.6$ mN/m we get $A_{LC} = 0.514$ nm$^2$ and $A_2 = 0.545$ nm$^2$.

The values of $A_{LC}$ at other temperatures can be obtained by interpolation from the dependencies $A = f(T)$ at $\pi_2 = 30.6$ mN/m. These and the values of $A_{LE}$ found from the kink in the $\pi/A$ isotherm are summarized in Table 1. Note that the comparison of the values of $A_{LC}$ with data obtained by Watts et al.[26] for the bilayers of phosphatidyl glycerol, and similar data for dimyristoyl phophatidyl glycerol (DMPG) reported by Pascher et al.[27] for the packing cross section of the crystalline lipid, were 0.48 and 0.44 nm$^2$ per molecule, confirming the reliability of our data.

The subsequent application of eq 1 yields the dependence $\Delta S_{tr} = f(T)$, which is also presented in Table 1. This quantity is related to the more frequently used $\Delta Q_{tr}$ in the following way

$$\Delta Q_{tr} = \Delta S_{tr} T = (S_{LC} - S_{LE})T \qquad (6)$$

Figure 7 shows that $\Delta Q_{tr}$ for DPPG monolayers increases linearly with increasing temperature. Many authors reported, however, a decrease of this parameter and a positive sign of the transition heat.[6,21,28,29] This can be explained as the consequence of selection of the initial and final state of the phase transition. In our case, the liquid-expanded state was chosen as initial, and the liquid-condensed state as final state, so that the values of $\Delta S_{tr}$ and $\Delta Q_{tr}$ are negative and differ from those of

**TABLE 1: Some Thermodynamic Parameters Describing the LE−LC Phase Transition in DPPG Monolayers Spread on a Subphase Modeling Biological Liquids**

| $T$, K | $A_{LC}$, nm²/molecule | $A_{LC}$, m²/mol | $A_{LE}$, nm²/molecule | $A_{LE}$, m²/mol | $\Delta S_{tr}$, J/mol·K | $\Delta Q_{tr}$, kJ/mol |
|---|---|---|---|---|---|---|
| 298 | 0.463 | 278819 | 1.014 | 610630 | −392 | −117 |
| 300 | 0.464 | 279421 | 0.956 | 575703 | −350 | −105 |
| 303 | 0.466 | 280625 | 0.880 | 529755 | −294 | −89 |
| 305 | 0.468 | 281830 | 0.826 | 497417 | −254 | −77 |
| 308 | 0.478 | 287852 | 0.810 | 487782 | −236 | −73 |
| 310 | 0.484 | 291465 | 0.776 | 467307 | −207 | −64 |
| 312 | 0.489 | 294476 | 0.721 | 434186 | −165 | −51 |
| 314 | 0.491 | 295680 | 0.690 | 415518 | −141 | −44 |

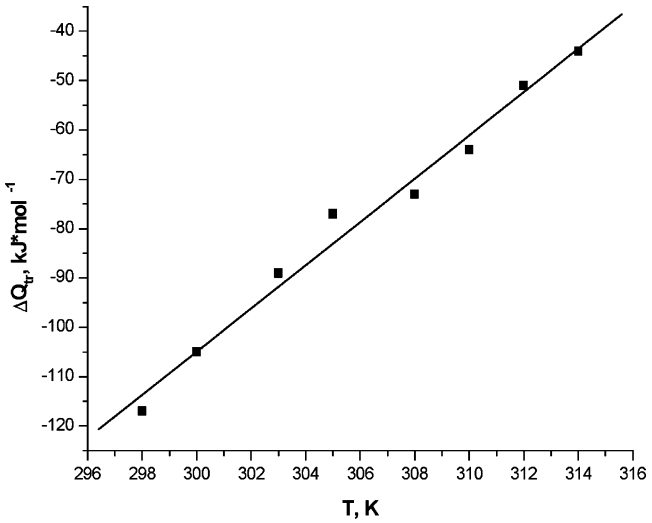


**Figure 7.** Transition enthalpy as a function of temperature: straight line, best linear fit.

other authors only in sign.[6,29] The values $\Delta Q_{tr}$ obtained here change from −71.9 to −42.5 kJ/mol in the temperature range between 308 and 314 K, respectively.

The extrapolation of the dependence $\Delta Q_{tr} = f(T)$ toward $\Delta Q_{tr} = 0$ allows us to determine the critical temperature $T_c$ beyond which there is no distinction between the two-dimensional phases, and the phase transition vanishes. For DPPG monolayers on a biosubstrate subphase, $T_c$ is 324 K (51 °C). Unfortunately, there are no data in the literature for a direct comparison. The deviation of our data for DPPG from those for other monolayers and bilayers may be probably explained by the presence of different ions in the subphase.[30,31]

## Conclusions

Under compression DDPG monolayers spread on a biologically relevant subphase show a LE−LC phase transition in the temperature range between 298 and 314 K. BAM images taken simultaneously demonstrate, however, that the monolayer in the transition region is highly inhomogeneous. This inhomogeneity is connected, probably, with the condensation effect of $Ca^{2+}$ ions in the subphase on the negatively charged lipid molecules. The opposite effect of $Na^+$ ions hinders a condensation in the monolayer before the plateau region. The fast growth of domains starts when the electrostatic repulsion caused by the presence of $Na^+$ ions is suppressed by the applied surface pressure. Consequently, their shapes become chaotic and the distribution within the monolayer is not uniform. The determination of monolayer parameters in the plateau region by processing of BAM images can yield, therefore, large errors. A data interpretation procedure is proposed here based only on the set of experimental $\pi/A$ isotherms recorded at different temperatures. First, the dependencies $A = f(T)$ in numerical form are obtained from the $\pi/A$ isotherms in a range between certain values $\pi_1$ and $\pi_2$ taken on the isotherm just after the onset of the phase transition and after the plateau region, respectively. Second, the dependencies $A = f(T)$ were fitted by a polynomial from which then the derivatives $(\partial A/\partial T)_\pi$ can be calculated. Finally, the pressure dependence of this derivative is numerically integrated and the entropy change at LE−LC phase transition $\Delta S_{tr}$ is obtained. The substitution of $\Delta S_{tr}$ into the Clausius−Clapeyron equation allows correction of the initial guess of $A_{LC}$. As a consequence, the accuracy of the determined $\Delta S_{tr}$ can be improved. The determination of the transition heat and its temperature dependence gives the critical temperature for the investigated system. The proposed treatment for a determination of the two-dimensional phase transition thermodynamics may be recommended for systems where the application of other methods fails. Certainly, the proposed method is restricted to systems demonstrating satisfactory homogeneity of the monolayer and regularity of the corresponding $\pi/A$ isotherm, especially just at the beginning of the phase transition.

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